SEIZURE-INDUCED CARDIOMYOPATHY: Benefit of atenolol

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i. Abstract

Epilepsy affects 1-2% of the population in New Zealand and is associated with an increased mortality rate of two to three times that of the general population. This thesis examines the effect of seizures on cardiac function, as it is hypothesised that seizure-induced activation of the sympathetic system produces electrographic (ECG) abnormalities, cardiac dysfunction and structural damage. Using a rodent model, seizures were induced using the excitotoxin, kainic acid, either subcutaneously or via an intrahippocampal drug cannula.

The first section of this study demonstrated that systemic kainic acid administration produced generalised seizure activity developing to status epilepticus. Kainic acid administration produced an immediate drop in heart rate (by 28%) associated with bradyarrhythmias. This was followed by a progressive increase in seizure severity which coincided with the development of tachycardia, QTc prolongation and T wave elevation. Heart rate variability analysis demonstrated that seizure activity resulted in significant changes in autonomic function. Prophylactic therapy with atenolol or clonidine attenuated seizure-induced ECG changes and preserved normal cardiac morphology.

The second half of this thesis used an improved model of seizure in which kainic acid was administered directly into the hippocampus to prevent possible systemic effects. The results in this study clearly demonstrated that seizures produced cardiac dysfunction, particularly changes in heart rate, QTc interval and blood pressure. Seizure-induced cardiac dysfunction resulted in significant structural damage as early as 48 hours which was still present up to 28 days after the original seizure induction. Assessment of autonomic function using various techniques demonstrated that seizures resulted in an increase in plasma noradrenaline levels and enhanced sympathetic dominance at 48 hours. The seizure-induced tachycardia which ensued resulted in the development of dilated cardiomyopathy with significant cardiac structural injury. The formation of cardiac micro-lesions and fibrotic deposition is suggested to contribute to left ventricular dysfunction and an increased susceptibility to arrhythmia induction. Intervention therapy with atenolol, 60 minutes post seizure induction, preserved cardiac function and structure. Importantly, atenolol reduced the susceptibility to arrhythmia onset, which has been reported as a contributor to sudden death in epilepsy. Interestingly, atenolol treatment during seizures also reduced EEG and behavioural score severity and protected the hippocampus from injury. Attenuation of seizure activity with diazepam did not reduce the extent of cardiac dysfunction. Diazepam-treated animals had significantly higher blood pressure, left ventricular dilation and
an increased susceptibility to arrhythmia induction. However, combination therapy with atenolol and diazepam, proved effective at protecting both the heart and brain following seizure activity.

This thesis has consistently demonstrated that atenolol administration (prophylactic or intervention) offers significant protection against seizure-induced cardiomyopathy. Atenolol, therefore, should be considered for clinical use, prophylactically in epilepsy or as a rescue intervention during status epilepticus. Importantly, this study clearly demonstrates that atenolol in combination with diazepam offers superior therapeutic benefit, over either monotherapy, in an animal model of status epilepticus.
ii. Acknowledgements

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iii. Contents

SEIZURE-INDUCED CARDIOMYOPATHY: Benefit of Atenolol \ 1
i. Abstract \ 1
ii. Acknowledgements \ 3
iii. Contents \ 4
iv. List of Figure \ 9
v. List of Tables \ 13
vi. List of Abbreviations \ 14
vii. Publications and Conference Proceedings \ 15

Chapter 1: Introduction \ 1

1.1. Epilepsy \ 2
  1.1.1. Pathophysiology \ 2
  1.1.2. Seizure Types \ 3
  1.1.3. Treatment of Epilepsy \ 3
  1.1.4. Electroencephalography \ 4

1.2. The Heart \ 5
  1.2.1. Physiology \ 5
  1.2.2. Autonomic Control \ 5
  1.2.3. Electrocardiogram \ 10
  1.2.4. Heart Rate Variability \ 11

1.3. Cardiac Abnormalities during Seizures \ 14
  1.3.1. Tachycardia \ 14
  1.3.2. Bradycardia \ 15
  1.3.3. ECG changes \ 15
  1.3.4. Structural cardiac damage \ 16
  1.3.5. Sudden unexpected death in epilepsy (SUDEP) \ 20

1.4. Kainic Acid Induced Excitotoxicity \ 22
  1.4.1. Glutamate and glutamate receptors \ 22
  1.4.2. Glutamatergic excitotoxicity \ 22
  1.4.3. Kainic acid induced seizures \ 24

1.5. Other seizure models \ 25
  1.5.1. Domoic acid \ 25
  1.5.2. Pilocarpine and Lithium \ 25
## Chapter 2: Cardiac dysfunction following sc KA-induced seizures

2.1. Introduction ................................................. 30

2.2. Methods .................................................. 32
   2.2.1. Materials ............................................. 25
   2.2.2. Animals .............................................. 32
   2.2.3. Experimental protocol ............................. 32
   2.2.4. Surgical implantation of telemetric transmitter .. 32
   2.2.5. Seizure induction and telemetric/behavioural recordings .. 33
   2.2.6. EEG analysis ...................................... 34
   2.2.7. ECG analysis ...................................... 34
   2.2.8. Morphological characterisation of myocardial injury ... 35
   2.2.9. Statistics .......................................... 35

2.3. Results .................................................. 36
   2.3.1. Seizure activity .................................... 36
   2.3.2. Cardiac function ................................... 36

2.4. Discussion .............................................. 43

## Chapter 3: Heart rate variability during sc KA-induced seizures

3.1. Introduction .............................................. 47

3.2. Methods .................................................. 49
   3.2.1. Materials ............................................. 49
   3.2.2. Animals .............................................. 49
   3.2.3. Experimental protocol ............................. 49
   3.2.4. Data Analysis ...................................... 49
   3.2.5. Statistics .......................................... 49

3.3. Results .................................................. 50
   3.3.1. Heart rate variability analysis in the rat ............ 50
   3.3.2. Pharmacological autonomic modulation ............. 54

3.4. Discussion .............................................. 59
Chapter 4: Sympatholytics in seizure-induced cardiomyopathy .............................................. 62

4.1. Introduction .................................................................................................................. 63

4.2. Methods ....................................................................................................................... 64
  4.2.1. Materials .................................................................................................................. 64
  4.2.2. Animals .................................................................................................................... 64
  4.2.3. Experimental Protocol ............................................................................................. 64
  4.2.4. Seizure induction and telemetric/behavioural recordings ........................................... 64
  4.2.5. Morphological characterisation of myocardial injury ................................................. 64
  4.2.6. Data analysis .......................................................................................................... 65

4.3. Results ......................................................................................................................... 66
  4.3.1. Seizure activity ......................................................................................................... 66
  4.3.2. Cardiac function ...................................................................................................... 68

4.4. Discussion .................................................................................................................... 74

Chapter 5: Cardiac dysfunction following ih KA-induced seizures .................................. 79

5.1. Introduction .................................................................................................................. 80

5.2. Methods ....................................................................................................................... 82
  5.2.1. Materials .................................................................................................................. 82
  5.2.2. Animals .................................................................................................................... 82
  5.2.3. Experimental Protocol ............................................................................................. 82
  5.2.4. Seizure ...................................................................................................................... 82
  5.2.5. Noradrenaline and cortisol plasma levels ............................................................... 84
  5.2.6. Echocardiography .................................................................................................. 85
  5.2.7. Arrhythmia risk ....................................................................................................... 86
  5.2.8. Histology and immunohistochemistry .................................................................. 86
  5.3.9. Data analysis and statistics ..................................................................................... 88

5.3. Results ......................................................................................................................... 90
  5.3.1. Seizure activity ......................................................................................................... 90
  5.3.2. ECG .......................................................................................................................... 90
  5.3.3. Vagal-sympathetic effect ......................................................................................... 94
  5.3.4. Noradrenaline and cortisol plasma levels ............................................................... 95
  5.3.5. Echocardiogram .................................................................................................... 95
  5.3.6. Arrhythmia risk ...................................................................................................... 97
  5.3.7. Histology and immunohistochemistry .................................................................. 98

5.4. Discussion .................................................................................................................... 103
Chapter 6: Therapeutic interventions in seizure-induced cardiomyopathy

6.1. Introduction

6.2. Methods

6.2.1. Materials

6.2.2. Animals

6.2.3. Experimental Protocol

6.2.4. Seizure induction

6.2.5. Troponin levels

6.2.6. Echocardiography

6.2.7. Arrhythmia risk

6.2.8. Histology and immunohistochemistry

6.2.9. Data analysis and statistics

6.3. Results

6.3.1. Seizure activity

6.3.2. Cardiac function

6.3.3. Cardiac immunohistochemistry and histology

6.3.4. Hippocampal immunohistochemistry

6.4. Discussion

Chapter 7: Final discussion and conclusions

7.1. Summary of results

7.2. Comparison of kainic acid seizure models

7.3. Adrenergic modulation in seizure

7.4. Adrenergic mediated cardiac dysfunction

7.5. Merits and limitations of non-invasion measures of autonomic function

7.6. Improvements and future directions

7.7. Final conclusion

Chapter 8: Appendices

8.1. Supporting data for intrahippocampal KA

8.2. Effects of pre-treatment with diazepam prior to KA

8.3. Prophylactic treatment with atenolol prior to intrahippocampal KA

8.4. Comparison of subcutaneous and intrahippocampal KA administration
8.5. Blood brain barrier.................................................................................. 161
8.6. Dose determination.................................................................................. 162

Chapter 9: Reference List................................................................................ 163
iv. List of Figures

**Chapter 1: Introduction** .................................................................................................................. 1
Figure 1.1. EEG trace from a seizing rat demonstrating the different frequency bands ........... 4
Figure 1.2. Cardiac structural and contraction pathways ................................................................. 6
Figure 1.3. Illustration of central autonomic control areas ............................................................. 7
Figure 1.4. Autonomic modulation of cardiac function ................................................................. 9
Figure 1.5. Cardiac myocyte and cellular pathways contributing to contraction ....................... 10
Figure 1.6. Example rat and human ECG trace ........................................................................... 10
Figure 1.7. Physiology systems involved in altering HR ................................................................. 12
Figure 1.8. Explaining frequency analysis of RR intervals ............................................................ 13
Figure 1.9. Cellular mechanism of excitotoxicity ......................................................................... 23
Figure 1.10. Chemical structure of domoic acid, kainic acid and glutamate .............................. 23
Figure 1.11. Hippocampal structure and regions ........................................................................... 24
Figure 1.12. Proposed mechanisms of seizure-induced cardiac dysfunction .............................. 28

**Chapter 2: Cardiac dysfunction following sc KA-induced seizures** ........................................... 29
Figure 2.3.1. Behavioural and EEG activity following subcutaneous KA .................................... 37
Figure 2.3.2. Heart rate following subcutaneous KA ................................................................. 37
Figure 2.3.3. QTc interval following subcutaneous KA ............................................................... 38
Figure 2.3.4. T wave amplitude following subcutaneous KA ....................................................... 38
Figure 2.3.5. Representative arrhythmia ECG traces following subcutaneous KA .................... 39
Figure 2.3.6. Effect of KA on heart rate and systolic blood pressure in rats ............................... 39
Figure 2.3.7. Representative heart demonstrating patchy micro-lesions ................................... 41
Figure 2.3.8. Histological evidence of seizure-induced cardiac injury ........................................ 42
Figure 2.4.1. Representative time matched EEG, ECG and behavioural recording ................. 44

**Chapter 3: Heart rate variability during sc KA-induced seizures** ............................................... 46
Figure 3.3.1. Heart rate in an untreated rat over 24 hours ............................................................ 50
Figure 3.3.2. Heart rate frequency spectrum analysis from a control rat .................................... 51
Figure 3.3.3. Individual RR intervals and spectral analysis following ipratropium ................. 52
Figure 3.3.4. Individual RR intervals and spectral analysis following atenolol ......................... 53
Figure 3.3.5. Atenolol or ipratropium pre-treatment on behavioural activity following sc KA ... 54
Figure 3.3.6. Atenolol or ipratropium pre-treatment on heart rate following sc KA
Figure 3.3.7. Effect of seizure on heart rate variability spectral analysis
Figure 3.3.8. Autonomic effect of seizures on heart rate variability

Chapter 4: Sympatholytics in seizure-induced cardiomyopathy
Figure 4.3.1. Pre-treatment with atenolol or clonidine on total behavioural following sc KA
Figure 4.3.2. Atenolol or clonidine on EEG and behavioural activity following sc KA
Figure 4.3.3. Pre-treatment with atenolol or clonidine on heart rate following sc KA
Figure 4.3.4. Pre-treatment with atenolol or clonidine on QTc interval following sc KA
Figure 4.3.5. Pre-treatment with atenolol or clonidine on the T wave following sc KA
Figure 4.3.6. Pre-treatment with atenolol or clonidine on HRV following sc KA
Figure 4.3.7. Left ventricular histology in atenolol-KA and clonidine-KA animals
Figure 4.3.8. ECG/EEG and behavioural changes during seizure induction

Chapter 5: Cardiac dysfunction following ih KA-induced seizures
Figure 5.2.1. Intrahippocampal drug cannula
Figure 5.2.2. Delegation of rats into respective treatment groups
Figure 5.2.3. Protocol used to determine cardiac vagal sympathetic effect
Figure 5.2.4. Representative M-mode echocardiogram
Figure 5.3.1. EEG and behavioural changes following intrahippocampal KA
Figure 5.3.2. Individual trace of EEG activity and heart rate over 18 hrs following ih KA
Figure 5.3.3. Effect of intrahippocampal KA on heart rate
Figure 5.3.4. Effect of intrahippocampal KA on the QTc interval
Figure 5.3.5. Effect of intrahippocampal KA on the T wave amplitude
Figure 5.3.6. Effect of intrahippocampal KA on systolic blood pressure
Figure 5.3.7. Effect of intrahippocampal KA on frequency analysis of HRV
Figure 5.3.8. Effect of seizures on vagal sympathetic balance
Figure 5.3.9. Effect of seizure activity on plasma noradrenaline and cortisol
Figure 5.3.10. Left ventricular dimensions post-KA as assessed by echocardiography
Figure 5.3.11. Left ventricular function post-KA as assessed by echocardiography
Figure 5.3.12. Latency to aconitine-induced arrhythmias following seizure
Figure 5.3.13. Quantification of cardiac injury following intrahippocampal KA
Figure 5.3.14. Micrographs of cardiac apoptotic cells
Figure 5.3.15. Micrographs of MSB stained myocardium ........................................... 99
Figure 5.3.16. Micrographs of cardiac fibrotic micro-lesions with macrophage infiltration ....... 100
Figure 5.3.17. Micrograph and quantification of the hippocampal apoptotic cells ............... 101
Figure 5.3.18. Micrograph and quantification of the hippocampal macrophage infiltration ....... 102

Chapter 6: Therapeutic interventions in seizure-induced cardiomyopathy ...... 107
Figure 6.1.1. GABAA receptor conformation with benzodiazepine (BZP) binding sites ........ 108
Figure 6.2.1. Delegation of rats into respective experimental groups ................................. 109
Figure 6.3.1. Effect of intervention therapy on behaviour following seizure ....................... 112
Figure 6.3.2. Intervention therapy on EEG activity following seizures ............................. 113
Figure 6.3.3. EEG and behavioural activity in seizures following intervention therapy .......... 114
Figure 6.3.4. Intervention therapy on heart rate following seizures ................................. 115
Figure 6.3.5. Intervention therapy on QTc interval following seizures .............................. 115
Figure 6.3.6. Intervention therapy on T wave amplitude following seizures ...................... 115
Figure 6.3.7. Intervention therapy on systolic blood pressure following seizures ............... 116
Figure 6.3.8. Effect of intervention therapy on frequency analysis of heart rate variability ....... 117
Figure 6.3.9. Intervention therapy on plasma troponin I levels following seizures ............... 118
Figure 6.3.10. Intervention therapy on left ventricular function following seizures ............. 119
Figure 6.3.11. Intervention therapy on left ventricular dimensions following seizures ........ 119
Figure 6.3.12. Effect of intervention on latency to aconitine-induced arrhythmias ............... 120
Figure 6.3.13. Effect of intervention on seizure-induced cardiac injury ............................. 121
Figure 6.3.14. Left ventricular structural damage following intervention therapy ............... 122
Figure 6.3.15. Example ventricular micrograph from a diazepam seizure rat ..................... 123
Figure 6.3.16. Hippocampal micrographs staining for apoptosis ...................................... 125
Figure 6.3.17. Hippocampal micrographs staining for microglia (CD11) ............................. 126
Figure 6.3.18. Micrographs of demonstrating hippocampal ApopTag positive cells ............. 127
Figure 6.3.19. Micrographs of demonstrating hippocampal CD11 positive cells ................. 128
Figure 6.4.1. Proposed effect of diazepam on myocyte function ..................................... 131

Chapter 7: Final discussion and conclusions ............................................................... 133
Figure 7.1. Ionotropic glutamatergic receptor activation of specific autonomic regions ....... 137
Figure 7.2. Adrenoceptor distribution and density in the rat hippocampus ....................... 138
Figure 7.3. Proposed mechanism of synaptic adrenergic modulation of glutamate receptors ... 140
Figure 7.4. Relationship between seizures, heart rate and neuronal damage .............................................. 140
Figure 7.5. Comparison of heart rate variability analysis and pharmacological denervation ......................... 143
Figure 7.6. Proposed mechanism of seizure-induced cardiomyopathy ....................................................... 149

**Chapter 8: Appendices** .......................................................................................................................... 150

Figure 8.1.1. Intrahippocampal KA administration (0.5-2 nmol) on cumulative behaviours .................. 151
Figure 8.1.2. Intrahippocampal KA administration (0.5-2 nmol) on behavioural score ......................... 152
Figure 8.1.3. Intrahippocampal KA administration (0.5-2 nmol) on heart rate ........................................ 152
Figure 8.1.4. Intrahippocampal KA administration (0.5-2 nmol) on QTc interval .................................. 153
Figure 8.1.5. Intrahippocampal KA administration (0.5-2 nmol) on T wave amplitude ....................... 153
Figure 8.1.6. Left ventricular structure in rats 48 hours following KA (0.5-2 nmol) ................................. 154
Figure 8.1.7. Confirmation of cannula placement ....................................................................................... 155
Figure 8.1.8. Correlation between HR and behavioural score following ih KA ........................................ 156
Figure 8.1.9. Correlation between HR and cardiac fibrosis or apoptosis following ih KA ..................... 156
Figure 8.1.10. Correlation between hippocampal apoptosis and HR/behavioural score following ih KA . 156
Figure 8.2.1. Effect of diazepam or saline on seizure activity and HR prior to sc KA ............................. 157
Figure 8.3.1. Effect of atenolol pre-treatment for three days prior to ih KA .............................................. 158
Figure 8.3.2. Left ventricular structure from atenolol pre-treated seizure animals (48 hr) ..................... 158
Figure 8.4.1. Comparison of sc (10 mg/kg) and ih (2 nmol) KA on HR and behaviour ......................... 159
Figure 8.4.2. EEG and behavioural scores following KA administration ............................................... 160
Figure 8.5.1. Amount of 14C-Sucrose in the ipsilateral and contralateral brain following KA ............... 161
v. List of Tables

Chapter 1: Introduction .................................................................................................................. 1
Table 1.1. Four categories of brain wave patterns ...................................................................... 5
Table 1.2. Mechanism of action and distribution of adrenergic receptors .............................. 8
Table 1.3. Effect of seizures of cardiac function ......................................................................... 18
Table 1.4. Heart rate variability changes during seizures .......................................................... 19

Chapter 2: Cardiac dysfunction following sc KA-induced seizures ........................................... 29
Table 2.2.1. Behavioural scores allocated to observed rat behaviours ................................... 34
Table 2.3.1. Left ventricular morphological features 48 hr post-seizure ................................... 40

Chapter 3: Heart rate variability during sc KA-induced seizures ................................................. 46
Table 3.4.1. Frequency domain analysis in rats ...................................................................... 60

Chapter 4: Sympatholytics in seizure-induced cardiomyopathy .................................................. 62
Table 4.3.1. Latency to seizure behaviour onset ........................................................................ 66
Table 4.3.2. Left ventricular structure in atenolol or clonidine rats post-KA (48 hr) .................... 71
Table 4.4.1. Reported effects of beta blockers on seizure generation in animal studies .......... 75
Table 4.4.2. Reported effects of clonidine on seizure induction in rodent models ................... 77

Chapter 5: Cardiac dysfunction following ih KA-induced seizures ............................................. 79
Table 5.2.1. Measurements of left ventricular function using echocardiography .................... 86
Table 5.2.2. Classes according to the Lambeth Conventions .................................................... 86
Table 5.3.1. Behavioural activity following KA seizures ......................................................... 90
Table 5.4.1. Comparison of current study indices against defined cardiomyopathies .............. 105

Chapter 6: Therapeutic interventions in seizure-induced cardiomyopathy ............................... 107
Table 6.3.1. Behavioural activity ............................................................................................... 112
Table 6.4.1. Reported effects of diazepam administration on heart rate .................................. 129

Chapter 7: Final discussion and conclusions .............................................................................. 133
Table 7.1. Summary of reported glutamatergic modulation of cardiac autonomic function .................. 136

Chapter 8: Appendices .................................................................................................................. 150
Table 8.4.1. Morphological features at post subcutaneous and intrahippocampal KA (48 hr) .... 159
Table 8.6.1. Human estimated dose calculation ....................................................................... 162
vi. List of Abbreviations

10N: vagal nerve
ACh: Acetylcholine
AED: Antiepileptic drugs
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionat
Amy: Amygdala
ANOVA: Analysis of variance
ANS: Autonomic nervous system
AV: Atrioventricular
BBB: Blood brain barrier
BP: Blood pressure
BZP: Benzodiazepine
CA: Cornu Ammonis
Ca²⁺: Calcium ion
eAMP: cyclic adenosine monophosphate
Cl⁻: Chloride ion
CNS: Central nervous system
CPS: Complex partial seizures
CPu: Caudate putamen of the striatum
CTC: Clonic-tonic convulsions
CVLM: Caudal ventrolateral medulla.
DG: Dentate gyrus
DMH: Dorsal medial hypothalamus;
DVN: Dorsal vagal nucleus.
ECG: Electrocardiography
EEG: Electroencephalography
EV: End ouvme
FLE: Frontal lobe epilepsy
GABA: γ-Aminobutyric acid
GEPR: Genetically epilepsy prone rats
GPCR: G-protein coupled receptors
GTCS: Generalised tonic clonic seizures
HBN: Hypercontracture band necrosis
HF: High frequency
HR: Heart rate
HRV: Heart rate variability
iHR: intrinsic heart rate
ip: intraperitoneal
iv: intravenous
IVS: Interventricular septum
K⁺: Potassium ion
KA: Kainic acid
LF: Low frequency
LSep: Lateral septum
LVID: Left ventricular internal dimension
LVPW: Left ventricularaposterior wall thickness
MSB: Martius scarlet blue
NA: noradrenaline
Na⁺: Sodium ion
NAm: Nucleus ambiguus;
NMDA: N-methyl-D-aspartate
NS: Not significant
NTS: nucleus of the solitary tract;
u: Normalised unit
PAG: Periaqueductal gray;
PBN: Parabrachial nucleus;
PDE: Phosphodiesterase
PKC: Protein Kinase C
po: Oral administration
Prefro. Cortex: Prefrontal cortex;
PTZ: Pentylenetetrazol
PVC: Premature ventricular contraction
PVN: Paraventricular nucleus;
QTc: Corrected QT interval
REM: Rapid eye movement
RVLM: rostral ventrolateral medulla;
SA: Sinoatrial
SDNN: Standard deviation of normal beats
SE: Status Epilepticus
SEM: Standard error of the mean
SUDEP: Sudden unexpected death in epilepsy
TLE: Temporal lobe epilepsy
VGCC: Voltage gated calcium channels
VGSC: Voltage gated sodium channels
VSE: Vagal sympathetic effect
WDS: Wet dog shakes
vii. Publications and Conference Preceding’s

Journal articles


Abstracts


Oral Presentation


Poster Presentation


Abstract retrieved from: https://web.psy.otago.ac.nz/awcbr/Programmes/AWCBRprogramme2013.pdf


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Chapter 1

Introduction. Seizure-induced cardiomyopathy
1.1. Epilepsy

Epilepsy is a common chronic neurological disorder where patients experience recurrent seizure activity (Banerjee et al., 2009). Epilepsy affects 1.4-3.3% of the population and is associated with an increased mortality rate of two to three times that of the general population (WHO, 2001; Hauser et al., 1991). Approximately 5-10% of the population will experience a seizure at some point in their life time, with up to 50% of these patients going on to develop epilepsy (Jallon et al., 2001; Krumholz et al., 2007; WHO, 2012). Evidence of seizure-induced cardiomyopathy has been reported in clinical and animal studies. In some patients with epilepsy, the cause of death is unknown, and this is termed sudden unexpected death in epilepsy (SUDEP).

1.1.1 Pathophysiology

Epileptic seizures are initiated by persistent neuronal hyperexcitability due to an imbalance between excitatory and inhibitory processes in the brain (Engelbourghs et al., 2000). The neurons undergo prolonged depolarisation associated with rapid firing of repeated action potentials, resulting in recruitment of adjacent neurons and spreading of electrical activity to other areas of the brain (Engelbourghs et al., 2000). Seizures can be generated by genetic and environmental factors which alter the properties of the neuronal membrane, such as abnormal channel functioning, alterations in the neuronal ionic micro-environment and irregular neurotransmitter activity (Chapman, 2000). Environmental factors, such as trauma, stroke, oxygen deprivation, tumours and infection, can cause abnormal cellular discharges by altering ion levels or channel function (Annegers et al., 1996; Dichter, 1997; Engelbourghs et al., 2000). Perinatal injury or hypoxia, as well as developmental disorders, can initiate seizures in children (WHO, 2001). Furthermore, there is a 2.5-fold increase in the risk of developing epilepsy in individuals who have a first-degree relative with epilepsy (Annegers et al., 1996).

\(\gamma\)-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter, which acts on ionotropic GABA\(_A\) receptors and metabotropic GABA\(_B\) receptors. Activated of GABA\(_A\) receptors enhances permeability to chloride (Cl\(^-\)), thereby increasing the neuronal threshold (Henderson et al., 2006). Epilepsy is associated with decreased GABA release, reduced GABA\(_A\) activity and a decline in glutamic acid decarboxylase activity, resulting in an imbalance in neuronal excitability (De Deyn & Macdonald, 1990; Engelbourghs et al., 2000). Glutamate is also strongly implicated in seizure generation and exerts its action by increasing sodium (Na\(^+\)) and calcium (Ca\(^{2+}\)) conductance (Chapman, 2000). At present no specific genetic mutation relating to enhanced glutamatergic
function has been linked to human epilepsy, although elevated glutamate levels have been reported in epileptic patients (van Gelder et al., 1980; Engelbourghs et al., 2000).

1.1.2 Seizure types
During a seizure, patients may exhibit a range of behavioural changes, from mild tremors or staring behaviours, through to a loss of consciousness and full body convulsions. Partial seizures occur in a localised brain region and are further split into simple or complex partial, depending on whether consciousness is maintained (McNamara, 1994; WHO, 2001; Banerjee et al., 2009). If the excitation spreads to other brain regions, patients can go on to develop secondary generalised seizure activity (Banerjee et al., 2009). During generalised seizures patients exhibit a loss of consciousness and convulsant activity is commonly observed. Generalised seizures can be further categorised into absent, myoclonic, tonic-clonic, tonic and clonic symptoms (WHO, 2001; Banerjee et al., 2009). Status epilepticus (SE) is a neurological emergency which is loosely defined as a single or multiple recurring seizures lasting a minimum of 30 minutes (De Lorenzo et al., 1995). However, there is now a general consensus that seizure duration exceeding 5–10 minutes should be treated as SE, because generalised tonic-clonic seizures usually cease within 3-5 minutes (Lowenstein & Alldredge, 1998; Shorvon et al., 2008). SE has a high mortality (10–30%), with death occurring predominately within 30 days of the initial convulsive activity (DeLorenzo et al., 1995; Hitiris et al., 2007).

1.1.3 Treatment of Epilepsy
Treatment of epilepsy is successful in up to 70% of cases, and in some patients, therapy can be ceased within five years with no relapse (WHO, 2012). The most commonly used class of antiepileptic drugs (AED) are sodium channel blockers, such as carbamazepine, phenytoin and valproate, which reduce Na\(^+\) influx, thereby increasing the neuronal threshold (Engelbourghs et al., 2000). This class of drugs can have potential cardiac side effects by altering the activity of cardiac sodium channels, resulting in drug induced cardiac dysfunction and arrhythmias (Tomson & Kenneback, 1997). Although rare, carbamazepine has been associated with conduction disorders, such as AV block and bradycardia, and even congestive heart failure (Kasarskis et al., 1992; Tomson & Kenneback, 1997; Timmings, 1998). Phenytoin treatment can result in atrial or ventricular conduction depression, ventricular fibrillation and decreased cardiac output (Barron, 1976; Earnest et al., 1983; McGovern et al., 1984; Tomson & Kenneback, 1997). Severe cardiotoxic reactions and fatalities have also been reported following phenytoin, particularly in elderly patients (Su et al., 2009). The adverse effects of sodium channel blockers may be
implicated in the development of seizure-induced cardiomyopathy. Drugs which enhance GABAergic transmission are effective for treating seizures, particularly in the emergency setting (Portela et al., 2014; Tasker, 1998). Benzodiazepines and barbiturates allosterically enhance the activity of the GABA<sub>A</sub> receptor, increasing Cl<sup>-</sup> conductance (Engelbourghs et al., 2000). Benzodiazepines are potent, fast-acting anti-convulsant drugs, therefore they are preferred as the initial therapy for the treatment of status epilepticus, particularly diazepam and lorazepam (Lowenstein & Alldredge, 1998; Working Group on Status Epilepticus, 1993).

1.1.4 Electroencephalography

Electroencephalography (EEG) is widely used to assess electrical activity in the brain as it is cheap, reliable and easy to use (Buzsaki et al., 2012). The EEG measures the electrical activity within the cortex and detects the electrographic spiking associated with seizure activity (Pillai & Sperling, 2006). In humans, a 12 lead EEG is used which allows for localisation of seizures, however the EEG scalp electrodes only sample one third of the cortex and seizures initiated within basal regions may be undetected (Pillai & Sperling, 2006). The use of the EEG helps to confirm a clinical diagnosis of epilepsy, provides information on the region and assists in determining drug therapy (Smith, 2005). In animal studies, EEG recordings can be taken from within the cortex. This allows for a clear recording subject to low noise interference, unlike surface recordings which are associated with electrical noise stemming from the dura, bone and scalp. However, the detail obtained from the cortical encephalogram is limited by the number of electrodes which can be implanted, thereby reducing the ability to localise the site of seizure generation. EEG activity can be assessed by quantifying the different frequency bands within an EEG trace (as represented in Figure 1.1; and described in Table 1.1; and).

![EEG Trace](image)

**Figure 1.1.** Representative electroencephalographic (EEG) trace from a seizing rat with an expanded version demonstrating the different frequency bands which constitute the EEG trace.
**Table 1.1.** Four categories of EEG brain wave patterns and corresponding behavioural responses

<table>
<thead>
<tr>
<th>Category</th>
<th>Frequency Range</th>
<th>Corresponding Behavioural Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta</td>
<td>1.25-4.5 Hz</td>
<td>Dreamless sleep and loss of body awareness.</td>
</tr>
<tr>
<td>Theta</td>
<td>4.75-6.75 Hz</td>
<td>REM sleep; increased production of catecholamines; deep meditation; emotional experiences</td>
</tr>
<tr>
<td>Alpha</td>
<td>7-12.5 Hz</td>
<td>Relaxation; light trace; super-learning; serotonin production</td>
</tr>
<tr>
<td>Beta</td>
<td>12.75-35 Hz</td>
<td>Concentration; arousal; alertness; cognition. Associated with anxiety, disease and sympathetic activity.</td>
</tr>
</tbody>
</table>

*REM: rapid eye movement; Klimesch et al., 1999; Petit et al., 2004*

### 1.2. The Heart

#### 1.2.1 Physiology

The heart is a fibromuscular four chamber organ which contracts rhythmically to pump blood through the circulatory system to the rest of the body (Figure 1.2). The right atria receives deoxygenated blood from the superior and inferior vena cava, while the left atria is supplied with oxygen rich blood through the pulmonary veins. The right and left ventricles pump blood to the lungs and the rest of the body, respectively. Coordinated contraction and relaxation of the heart is critical to ensuring that the whole body receives an adequate blood supply for normal functioning (Figure 1.2). The sinoatrial (SA) node is a small mass of modified cardiac cells which act as the pacemaker of the heart (Kundu et al., 2000). The SA node is located in the right atrial epicardium, near the opening of the superior vena cava, and initiates the impulses which depolarise the rest of the heart, to produce contraction (Kundu et al., 2000; Catalano, 2002). These impulses are propagated along the internodal and intra-atrial tracts, to activate the atrioventricular (AV) node at the base of the right atria (Catalano, 2002). The bundle of His and Purkinje fibres carry the electrical activity from the AV node to the ventricles, allowing the ventricles time to contract (Kundu et al., 2000). It is important that co-ordinated contraction occurs to ensure sufficient blood is supplied to vital organs.

#### 1.2.2 Autonomic control

The autonomic nervous system (ANS) maintains homeostasis through regulation of heart rate (HR), respiration, digestion, micturition and reproduction (Jansen & Lagae, 2010). The ANS consists of two main subsystems, the sympathetic and parasympathetic nervous systems, and is controlled by medullary reflexes and the cerebral cortex (Shields, 1993; Jansen & Lagae, 2010). Both systems are tonically active and operate in conjunction with each other to control and maintain homeostasis between vital organs of the body, such as the heart, lungs, liver, and kidneys (Figure 1.3; Iversen et al., 2000).
Normal functioning of the ANS involves a complex interaction between many brain regions. The central ANS involves the cortical limbic areas, including the amygdala, insula cortex and cingulate cortex (Figure 1.3; Devinsky, 2004). These areas are connected with subcortical regions such as the hypothalamus, periaqueductal grey, parabrachial region in the pons, nucleus of the solitary tract (NTS) and ventrolateral medulla (Figure 1.3; Shields, 1993; Devinsky, 2004; Leung et al., 2006; Jansen and Lagae, 2010; Jehi, 2010).

The sympathetic nervous system is involved in the “fight or flight” response by increasing arousal and energy production, while inhibiting gastrointestinal function. Activation of the sympathetic system elevates HR, renin release and pupil dilation, as well as increases blood flow to lungs and skeletal muscle (Janig & McLachlan, 1992; Shields, 1993; Vaseghi & Shivkumar, 2008). Noradrenaline is the main neurotransmitter responsible for mediating sympathetic modulation. Sympathetic activity is further enhanced by innervation of the adrenal medulla, causing secretion of adrenaline (and noradrenaline) directly into the bloodstream allowing for widespread sympathetic discharge (Shields, 1993; Vaseghi & Shivkumar, 2008).
Figure 1.3. Illustration of central autonomic control areas (blue), including the regions involved in modulation of the cardiovascular system (maroon). NTS: nucleus of the solitary tract. Adapted from Green and Paterson, 2008; Cersosimo and Benarroch, 2013.
Noradrenaline and adrenaline activate G-protein coupled (GPCR) $\alpha_{1(2)}$ or $\beta_{1(3)}$ adrenoceptors, which are widely spread throughout the cardiovascular system (Table 1.2). Activation of $\beta_1$ and $\beta_2$ adrenoceptors increases intracellular levels of cyclic adenosine monophosphate (cAMP), which stimulates protein kinases and elevates intracellular $Ca^{2+}$ levels (Vaseghi & Shivkumar, 2008). $\alpha_1$ adrenoceptors are Gq coupled receptors linked to activation of phospholipase C, increasing intracellular $Ca^{2+}$ levels. $\alpha_1$ and $\beta_2$ adrenoceptors are responsible for vasoconstriction in gastrointestinal organs, and relaxation of vascular smooth muscle in skeletal muscle, respectively (Shields, 1993). $\beta_1$ (and to a lesser extent $\beta_2$) adrenoceptors predominately mediate sympathetic control of the heart by causing positive inotropic (force of contraction) and chronotropic (rate of contraction) effects (Shields, 1993a). $\alpha_2$ adrenoceptors are generally presynaptic and produce inhibitory actions by decreasing the production of cAMP, thereby reducing noradrenaline release (Shields, 1993; Vaseghi & Shivkumar, 2008).

Table 1.2. Mechanism of action and distribution of adrenergic receptors.

<table>
<thead>
<tr>
<th>Main distribution</th>
<th>G Protein</th>
<th>2nd messenger</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$</td>
<td>G$q$</td>
<td>DAG and IP$_3$</td>
<td>Smooth muscle contraction</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>G$i$</td>
<td>↓cAMP</td>
<td>Autoreceptor (decrease noradrenaline release)</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>G$s$</td>
<td>↑cAMP</td>
<td>Increase HR, contractility, automaticity,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>conduction, renin release, lipolysis</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>G$s$</td>
<td>↑cAMP</td>
<td>Smooth muscle relaxation, glyconeogenesis,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>increased cardiac output, insulin secretion</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>G$s$</td>
<td>↑cAMP</td>
<td>Lipolysis, smooth muscle relaxation, vasodilation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>coronary arteries, negative cardiac inotropy</td>
</tr>
</tbody>
</table>

$cAMP$: cyclic adenosine monophosphate, CNS: central nervous system, DAG: diacylglycerol, IP$_3$: Inositol trisphosphate

Activation of the parasympathetic nervous system promotes the “rest and digest” response, by increasing gut motility, salivation and micturition (Janig & McLachlan, 1992; Wess, 1996; Gerber et al., 2001; Bymaster et al., 2002). Acetylcholine (ACh) controls parasympathetic activation by binding to muscarinic (M$_1$-$5$) receptors. M$_2$ receptors are present in the ventricular myocardium where they act to decrease contraction of the heart through reduced cAMP production (Figure 1.5; Janig & McLachlan, 1992; Caulfield, 1993; Bymaster et al., 1999; Vaseghi & Shivkumar, 2008). In comparison, M$_3$ receptors are located on vascular endothelial cells where they increase synthesis of nitric oxide, leading to vasodilation.
The sympathetic and parasympathetic systems innervate the heart and controlling cardiac rate and force of contraction (Figure 1.4). Activation of the sympathetic system results in increased HR and force of contraction through activation of β adrenergic receptors. This is primarily mediated by β₁-adrenoceptors as they represent 77-86% of β receptors expressed in human myocardium (Vaseghi & Shivkumar, 2008). Stimulation of β adrenoceptors in the myocardium also leads to activation of the voltage-dependent L-type Ca²⁺ channels (Figure 1.5). The resultant Ca²⁺ influx into the myocytes evokes the intracellular release of Ca²⁺ from within the sarcoplasmic reticulum to promote ventricular and atrial contraction, as well as an increase in cardiac output (Figure 1.5; Iversen et al., 2000; Vaseghi & Shivkumar, 2008). Parasympathetic activation has negative ionotropic and chronotropic effects via the release of ACh by the vagus nerve. ACh activates muscarinic M₂ receptors at the SA and AV nodes. This action causes a reduction in Ca²⁺ levels, and an increase in K⁺ influx, thereby hyperpolarising the myocardium and reducing both the force and rate of contraction (Figure 1.5; Sundaram et al., 1989; Tsai et al., 2005).
1.2.3 Electrocardiogram

The electrocardiogram (ECG) is a graphical representation of the electrical changes which occur within the entire heart over one heart beat and is used to assess cardiac function (Figure 1.6). The P wave represents the discharge of the SA node and contraction of the left and right atria (Kundu et al., 2000; Stouffee, 2009). Factors which prolong impulse propagation in the atria (such as fibrosis or hypertrophy) will lengthen the duration of the P wave (Stouffee, 2009). The QRS complex represents the depolarisation of both ventricles (Catalano, 2002). Prolonged QRS duration can occur due to myocardial infarction (MI), hypokalemia, severe bradycardia or His-Purkinji system disease (Marsh et al., 2008; Stouffee, 2009). The T wave of the ECG is the final wave and it corresponds to the relaxation of the ventricles (Kundu et al., 2000; Catalano, 2002). The amplitude can be increased by structural damage such as left or right ventricular hypertrophy or hypertrophic cardiomyopathy (Marsh et al., 2008; Stouffee, 2009). The QT interval is used as a marker of arrhythmia risk as it represents ventricular depolarisation and repolarisation. Structural damage or impaired electrical conductance can alter the ventricular contraction/relaxation cycle, thereby increasing the risk of fatal arrhythmias such as ventricular fibrillation (Rajan & Zellweger, 2004). In the rodent ECG there is an absence of an isoelectric
interval between the S wave and the T wave due to the involvement of different ventricular repolarisation mechanisms (Figure 1.6). In human myocardium, potassium channels, such as the inward K+ rectifier and delayed K+ rectifier currents mediate repolarisation, however in rodents the primary current involved in repolarisation is the rapidly activating and inactivating 4-aminopyridine-sensitive transient outward current (Gussak et al., 2000; Keating & Sanguinetti, 2001; Antzelevitch, 2006).

![Figure 1.6](image)

**Figure 1.6.** ECG for a single heartbeat. Left image shows a human ECG trace and the right image represents a rat ECG trace with altered T wave.

### 1.2.4 Heart Rate Variability

Heart rate variability (HRV) is a measure of the variation in consecutive heart beats over time (Naritoku et al., 2003; Acharya et al., 2006). Normal variation in the HR is mediated by autonomic regulation of the heart and circulatory system (Figure 1.7) (van Ravenswaaij et al., 1993; Naritoku et al., 2003; Acharya et al., 2006). HRV is a non-invasive index of autonomic activity, which is easy to perform and is highly reproducible (Acharya et al., 2006). The degree of variability in the HR provides information about the functioning of the nervous system and the ability of the heart to respond (Acharya et al., 2006). The sinus rhythm exhibits fluctuations around the mean heart rate because of continuous changes in the sympathetic-parasympathetic balance. Frequent small adjustments in heart rate are made by cardiovascular control mechanisms, resulting in periodic fluctuations in heart rate (Figure 1.7). The main fluctuations are respiratory sinus rhythm and baroreflex-or thermoregulation-related heart rate changes (van Ravenswaaij et al., 1993). HRV can be determined by time domain analysis or spectral frequency domain analysis of RR intervals (van Ravenswaaij et al., 1993). Both of these methods require accurate measuring of the R waves and can be performed on short segments (0.5 to 5 minutes) or 24 hour ECG recordings (van Ravenswaaij et al., 1993).
Figure 1.7. Systems involved in regulation of cardiac output include parasympathetic (green) and sympathetic (red) modulation of heart rate, as well as other non-autonomic physiological systems (purple). RAAS: renin angiotensin aldosterone system.
1.2.2.1 Time domain analysis

Time domain analysis measures beat-to-beat changes in HR based on the RR intervals during an ECG recording of 0.5-5 minutes or 24 hours (van Ravenswaaij et al., 1993). SDNN is the standard deviation of all normal RR intervals measured between consecutive sinus beats and is usually performed as a measure of short-term variability, although 24 hour traces can also be informative (van Ravenswaaij et al., 1993; Tsuji et al., 1994; Bilchick & Berger, 2006). It is important when comparing SDNN values that the sampling time used is consistent as a longer recordings, particularly 24 hour traces, will naturally have greater variability.

1.2.2.2 Frequency domain analysis

The advantage of spectral analysis is that it is possible to study the total amount of variability as well as the oscillation frequency (how much the HR fluctuates per second; van Ravenswaaij et al., 1993). Spectral analysis involves decomposing the series of sequential RR intervals into a sum of sinusoidal functions of different amplitudes and frequencies by the Fourier transform algorithm (Figure 1.8; van Ravenswaaij et al., 1993). The low frequency band (0.04-0.15 Hz) is associated with both sympathetic and parasympathetic modulation, whereas the high frequency band (0.15-0.4 Hz) is controlled almost exclusively by parasympathetic and respiratory effects (Bilchick & Berger, 2006). Sympathetic activation is a slow modulatory effect (low frequency), as the action of noradrenaline on the SA node is terminated when noradrenaline is removed from the synapse by noradrenaline reuptake transporters. Vagal transmission is limited by the presence of cholinesterases within the SA node which rapidly hydrolyse ACh. This action produces a rapid but brief parasympathetic effect and therefore is associated with high frequency fluctuations in HR (Akselrod et al., 1985; Stein et al., 1994; Cumm et al., 1996; Stauss, 2003). The ratio of LF to HF power is used to determine the sympathovagal balance (Montano et al., 1994; Bilchick & Berger, 2006). A problem with frequency domain analysis is that if RR intervals are deleted or edited the power density can be reduced or amplified at some frequency components, therefore making it important to replace any missing RR intervals (Bilchick & Berger, 2006). HRV, as determined by frequency domain analysis, is useful as a means of examining the balance between the sympathetic and parasympathetic systems following seizure activity.
1.3. Cardiac Abnormalities during Seizures

Seizures have been increasingly associated with cardiac injury in both clinical and animal studies. Epileptiform activity has been increasingly associated with changes in autonomic function, particularly on the cardiorespiratory system, leading to cardiac arrhythmias, apnoea and hypoxia (Provini et al., 1999; Jansen and Lagae, 2010).

1.3.1 Tachycardia

Tachycardia (>100 b.p.m.) is commonly reported, occurring in 33-100% of seizures, and may precede the EEG onset of the seizure by 0.7 to 49.3 sec (Table 1.3; Opherk et al., 2002; Surges et al., 2009b; Jansen & Lagae, 2010). Patients with epilepsy have a higher resting HR than the general population due to elevated sympathetic activity or lower parasympathetic control (Harnod et al., 2008). Uncorrected tachycardia has the potential to cause long-term myocardial damage.
and ventricular tachyarrhythmias with fatal consequences which may contribute to the incidence of sudden death in epilepsy (Jansen & Lagae, 2010). Generalised tonic-clonic seizures (GTCS) are associated with the highest HRs (Nei et al., 2000; Wilder-Smith & Lim, 2001; Opherk et al., 2002; Leutmezer et al., 2003). Ventricular tachyarrhythmias account for 90% of sudden cardiac deaths, and are generally caused by increased sympathetic tone and structural damage (Du et al., 1995; Schuele et al., 2007).

1.3.2 Bradycardia

Ictal bradycardia (<40 b.p.m.) is rarely reported, occurring in fewer than 2% of seizures, however it should not be dismissed as a contributor to sudden death (Nei et al., 2000; Leutmezer et al., 2003; Devinsky, 2004). Bradycardia leading to asystole of more than 10 seconds could impose severe hypoxaemia resulting in a fatal decrease in cerebral oxygen supply (Surges et al., 2009b; Sevcencu & Struijk, 2010). Bradycardia is more prevalent in seizures of temporal or frontal lobe origin, and may be more frequent in patients with left-sided foci (Oppenheimer et al., 1991; Devinsky, 2004; Jansen & Lagae, 2010). Ictal bradycardia tends to start 10-30 seconds after the EEG onset of the seizure (Sevcencu & Struijk, 2010). The incidence of bradycardia may be underestimated, Zijlmans et al. (2002) reported a decrease in HR of at least 10 b.p.m. in 7% of seizures (15% of patients). A Singapore based study of partial seizures also reported a high incidence of sinus bradycardia occurring in 16% of seizures (Wilder-Smith & Lim, 2001). In a United Kingdom study, twenty epileptic patients with refractory partial seizures were implanted with a loop ECG recorder for 24 months (Rugg-Gunn et al., 2004). Ictal bradycardia was reported in 35% of patients (2.1% of seizures), and four patients (21%) required a permanent pacemaker due to bradycardia or asystole. Asystole has been reported to occur in 0.27-1.2% of patients with epilepsy (up to 2.3% of seizures), with the majority of these reported in seizures of a temporal lobe origin (Nei et al., 2000; Scott & Fish, 2000; Zijlmans et al., 2002; Rocamora et al., 2003; Rugg-Gunn et al., 2004; Schuele et al., 2007).

1.3.3 ECG changes

ECG abnormalities are more common in refractory and temporal lobe epilepsy (TLE), where up to 40-60% of patients have been reported to have at least one ictal rhythm or repolarisation abnormality (Table 1.3; Opherk et al., 2002; Zijlmans et al., 2002; Nei et al., 2004; Stollerberger & Finsterer, 2004; Surges et al., 2009b; Devinsky, 2004). Excessive autonomic stimulation can cause structural heart damage, increasing susceptibility to cardiac arrhythmias or ischaemia
Cardiac arrhythmias are caused by malfunctioning of cardiac electrophysiology, such as abnormal channel functioning or tissue damage. ECG abnormalities have been reported in 35% of seizures and 72% of epileptic patients (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002; Devinsky, 2004; Nei et al., 2004; Stollerberger & Finsterer, 2004; Surges et al., 2009a; Surges et al., 2009b). These include atrial fibrillation, supraventricular tachycardia, ventricular premature depolarisation, branch block and first degree AV block (Nei et al., 2000; Devinsky, 2004). Most changes are benign, however potentially serious abnormalities (such as ST depression and T wave inversion) have been reported to occur in 6 to 14% of seizures (Opherk et al., 2002; Devinsky, 2004).

The QT interval (Figure 1.6) is used as a clinical index of ventricular repolarisation (Whisel et al., 2001). A prolonged QT interval is an established risk factor for life-threatening ‘Torsade de Pointes’ tachycardia and ventricular arrhythmias (Shimizu & Antzelevitch, 1998; Metcalf et al., 2009a; Surges et al., 2010). Prolongation of the QT interval can be caused by enhanced sympathetic stimulation and abnormal channel functioning, such as inhibition of outward K+ currents or enhancement of inward Na+ or Ca2+ currents (Tavernor et al., 1996; Shimizu & Antzelevitch, 1998; Yan et al., 2003). Even a transient increase as observed during epileptic discharge, can predispose a patient to ventricular fibrillation (Dasheiff, 1991; Drake et al., 1993; Tavernor et al., 1996). In 68% of temporal lobe seizures the QT interval is shortened, particularly in patients with right sided seizure onset. A shortening of the QT interval can be caused by hyperkalaemia, hypercalcaemia or acidosis (Surges et al., 2010). This rapid repolarisation can facilitate re-entrant excitation, leading to atrial fibrillation (Surges et al., 2010). It is therefore important to be aware of the development of ictal ECG abnormalities as these can help identify those patients at risk of sudden cardiac death. The presence of AV block, intraventricular conduction defects, QT prolongation and a resting HR of greater than 90 b.p.m.; are all markers of sudden cardiac death (Dasheiff, 1991; Zipes & Wellens, 1998).

1.3.5 Structural cardiac damage

Elevated catecholamine levels during seizures have been associated with the development of ischaemic micro-infarcts and micro-lesions leading to cardiac decompensation and arrhythmias (Boggs et al., 1993; Kloster & Engelskjon, 1999; Manno et al., 2005; Vaseghi & Shivkumar, 2008). Fibrosis of the walls of small coronary arteries, interstitial myocardial fibrosis, atrophy of cardiomyocytes, myofilament degeneration, subendocardial fibrosis, leukocytic infiltration and oedema of conductive tissue have been described at post-mortem in hearts from epileptics (Stollerberger & Finsterer, 2004). Natelson et al. (1998) found pathological changes present in
five out of seven hearts from epileptic patients. Four hearts exhibited evidence of irreversible perivascular and interstitial fibrosis, and all had reversible myocyte vacuolisation, predominantly in the subendocardium (Natelson et al., 1998). Repeated hypoxaemia and increased catecholamines during seizure can cause myocardial ischaemia, QT-lengthening and interictal changes in heart rate variability (Kloster & Engelskjøn, 1999; Ryvlin, 2006; Jansen & Lagae, 2010). Seizure-induced structural heart damage makes the heart more susceptible to fatal arrhythmias, increasing the risk of sudden cardiac death.

1.3.6 Autonomic nervous system and seizures

Seizures which arise from or spread to areas in the central autonomic network can mimic or alter autonomic effects (Devinsky, 2004; Jansen & Lagae, 2010). Seizure activity generally results in enhanced sympathetic stimulation such as tachycardia (HR>100 b.p.m.), tachypnoea, hypertension, pupil dilation, diaphoresis and facial flushing. This can lead to seizure-induced cardiovascular dysfunction, pulmonary oedema and postictal depression of autonomic respiratory reflexes (Devinsky, 2004). Provini et al. (1999) reported that autonomic activation was a common symptom of nocturnal frontal lobe epilepsy, with tachycardia occurring in 88% of seizures and changes in respiratory rhythm in 77% of the cases. Ictal parasympathetic activity or sympathetic inhibition produces symptoms such as increased salivation, gastric acid secretion and peristalsis, as well as decreased heart (HR <40 b.p.m.), respiratory rates and reduced blood pressure (BP; Devinsky, 2004).

Epilepsy associated with autonomic neuronal dysfunction, produces abnormalities in HR as well as a loss of RR variability and decreased high frequency spectral band power (Naritoku et al., 2003; Harnod et al., 2008). Frequency analysis of patients with TLE demonstrates altered cardiac innervation with all studies reporting changes in power, and reduced overall variability (SDNN) when compared to controls (Table 1.4). Both sympathetic (Druschky et al., 2001; Ansakorpi et al., 2002; Dutsch et al., 2006; Li et al., 2006; Shobha et al., 2007; Dericioglu et al., 2013) and parasympathetic (Massetani et al., 1997; Tomson et al., 1998; Ansakorpi et al., 2004; Ponnusamy et al., 2011) dominance have been reported in patients with TLE. Generalised tonic-clonic seizures are associated with sympathetic dominance, as determined by increased LF power (Vaughn et al., 1996; Evrengul et al., 2005; Hattori et al., 2007) and/or reduced vagal HF power (Evrengul et al., 2005; Dutsch et al., 2006; Harnod et al., 2008; Harnod et al., 2009; Mativo et al., 2010).
## Table 1.3. Summary of the effect of seizures on heart rate and ECG activity.

<table>
<thead>
<tr>
<th>Seizure</th>
<th>Tachycardia</th>
<th>Bradycardia</th>
<th>ECG</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keilson et al., 1989</td>
<td>Various</td>
<td>98%</td>
<td>-</td>
<td>16% had HR changes preceding the seizures</td>
</tr>
<tr>
<td>Vaughn et al., 1996</td>
<td>Various</td>
<td>57%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Tigaran et al., 1997</td>
<td>Various</td>
<td>-</td>
<td>8%</td>
<td>62% has ECG changes suggesting ischaemic damage</td>
</tr>
<tr>
<td>Schernthaner et al., 1999</td>
<td>Various</td>
<td>82.5%</td>
<td>3.3%</td>
<td>HR changes occurred prior to seizure onset in 76.1%</td>
</tr>
<tr>
<td>Garcia et al., 2001</td>
<td>Various</td>
<td>86%</td>
<td>-</td>
<td>HR changes more common in temporal seizures</td>
</tr>
<tr>
<td>Opherk et al., 2002</td>
<td>Various</td>
<td>73% total 100% of GTCS</td>
<td>-</td>
<td>&gt;25 y had greater ↑ in HR; hemisphere had no effect. TLE higher risk of ECG changes</td>
</tr>
<tr>
<td>Leutmezer et al., 2003</td>
<td>Various</td>
<td>87%</td>
<td>1.4%</td>
<td>Increase more common in TLE and right-sided</td>
</tr>
<tr>
<td>Woodruff et al., 2003</td>
<td>Various</td>
<td>120.6 b.p.m.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Nei et al., 2004</td>
<td>Various</td>
<td>94%</td>
<td>-</td>
<td>Higher HR in the SUDEP group</td>
</tr>
<tr>
<td>O'Regan &amp; Brown, 2005</td>
<td>Various</td>
<td>35% of focal, 57% of GTCS</td>
<td>13% of focal, 19% of GTCS</td>
<td>↑ in HR as great as 2.2-fold</td>
</tr>
<tr>
<td>Brotherstone et al., 2010</td>
<td>Various</td>
<td>-</td>
<td>23% QTc prolongation</td>
<td></td>
</tr>
<tr>
<td>Moseley et al., 2011</td>
<td>Various</td>
<td>57%</td>
<td>2%</td>
<td>Tachycardia common in GTCS. QTc prolongation were more common in CN in the partial seizure group.</td>
</tr>
<tr>
<td>Galimberti et al., 1996</td>
<td>Partial</td>
<td>72%</td>
<td>26%</td>
<td>Bradycardia more common in TLE (80%)</td>
</tr>
<tr>
<td>Rugg-Gunn et al., 2004</td>
<td>Partial</td>
<td>80%</td>
<td>2.1%</td>
<td></td>
</tr>
<tr>
<td>Blumhardt et al., 1986</td>
<td>TLE</td>
<td>92%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Li et al., 1995</td>
<td>TLE</td>
<td>39% (139 b.p.m.)</td>
<td>5%</td>
<td>Greater ↑ HR in patients over 25 yo</td>
</tr>
<tr>
<td>Masetani et al., 1997</td>
<td>TLE</td>
<td>67% (143 b.p.m.)</td>
<td>-</td>
<td>Lower LF and HF values in the epilepsy group</td>
</tr>
<tr>
<td>Novak et al., 1999</td>
<td>TLE</td>
<td>HR of 140 b.p.m.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Surges et al., 2010</td>
<td>TLE with GTCS</td>
<td>&gt;100 b.p.m. during GTCS</td>
<td>76% had benign arrhythmias. QTc prolongation (12%) and shortening (68%)</td>
<td>Duration of seizures had no effect on HR QTc shortening- more frequent in females &amp; right side onset</td>
</tr>
<tr>
<td>Devinsky, 1985</td>
<td>CPS</td>
<td>17%</td>
<td>17%</td>
<td>83% patients had angina-like pain</td>
</tr>
</tbody>
</table>

CPS: Complex partial seizures; GTCS: generalised tonic-clonic seizures; HF: high frequency (parasympathetic activity); HR: heart rate; LF: low frequency (sympathetic and parasympathetic activity); TLE: Temporal lobe epilepsy.
<table>
<thead>
<tr>
<th>Seizure type</th>
<th>HR</th>
<th>SDNN</th>
<th>LF</th>
<th>HF</th>
<th>Unit</th>
<th>LF/HF</th>
</tr>
</thead>
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<tr>
<td><strong>Sympathetic dominance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLE (Druschky et al., 2001)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>bpm2</td>
<td>↑</td>
</tr>
<tr>
<td>TLE (Ansakorpi et al., 2002)</td>
<td>NS</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>ms2</td>
<td>↑</td>
</tr>
<tr>
<td>TLE (Ferri et al., 2002)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>bpm2</td>
<td>↑</td>
</tr>
<tr>
<td>TLE (Ansakorpi et al., 2002)</td>
<td>NS</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>ms2</td>
<td>↑</td>
</tr>
<tr>
<td>TLE (Evrengul et al., 2005)</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>nu</td>
<td></td>
</tr>
<tr>
<td>TLE (Ferri et al., 2002)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>nu</td>
<td>↑</td>
</tr>
<tr>
<td>TLE (Calandra-Buonaura et al., 2012)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td><strong>Parasympathetic dominance</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TLE (Massetani et al., 1997)</td>
<td>↑</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>ms2</td>
<td>↓</td>
</tr>
<tr>
<td>Myoclonic epilepsy (Tomson et al., 1998)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>ms2</td>
<td>↓</td>
</tr>
<tr>
<td>TLE (Yildiz et al., 2011)</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>ms2</td>
<td>↑</td>
</tr>
<tr>
<td>TLE (Ansakorpi et al., 2004)</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Refractory epilepsy (Ponnusamy et al., 2011)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Various (Brotherstone &amp; McLellan, 2012)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>↑</td>
</tr>
<tr>
<td><strong>No change in autonomic balance</strong></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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</tr>
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<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<td>NS</td>
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</tr>
<tr>
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<td>↑</td>
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<td>NS</td>
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<td></td>
</tr>
<tr>
<td>Various (Persson et al., 2007)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Refractory generalised (Harnod et al., 2008)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
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<tr>
<td>Various (Hallioglu et al., 2008)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>FLE and GTCS (Harne et al., 2009)</td>
<td>↑</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Refractory TLE (Mukherjee et al., 2009)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>SUDEP (Surges et al., 2009a)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Various (Raju et al., 2012)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Refractory epilepsy (Stavrinou et al., 2014)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

**NS**: not significantly different, **TLE**: Temporal lobe epilepsy, **FLE**: Frontal lobe epilepsy, **SUDEP**: Sudden unexpected death in epilepsy, **GTCS**: generalised tonic-clonic seizures. Heart rate variability measured as normalised units (nu) or as raw data where power was determined from the tachograph as heart rate (bpm²) or RR interval (ms²).
1.3.7 Sudden Unexpected Death in Epilepsy (SUDEP)

Epilepsy is associated with an increased mortality rate compared to the general population (Lhatoo et al., 2001). In 7 to 17% of epileptics the cause of death is sudden and unexpected (SUDEP; Sperling, 2001; Opherk et al., 2002; Evrengul et al., 2005; Schuele et al., 2007). SUDEP is defined as “sudden, unexpected, witnessed or unwitnessed, non-traumatic, and non-drowning death in patients with epilepsy, with or without evidence of a seizure, and excluding documented SE, in which post-mortem examination does not reveal a toxicological or anatomic cause for death” (Nashef & Shorvon, 1997). ‘Definite SUDEP’ must meet all the criteria including post-mortem examination which has failed to establish a cause of death, whereas ‘probable SUDEP’ meets the criteria without an autopsy (Annegers et al., 1998; Sperling, 2001). SUDEP is the most common epilepsy-related cause of death, particularly in patients with chronic epilepsy. Ictal cardiorespiratory alterations are likely to be involved in the pathophysiology of SUDEP, including tachy- and brady- arrhythmias, as well as hypoventilation pulmonary oedema (Tomson et al., 2008). Studies have shown, younger patients (20-40 years), have a 24-fold higher risk of sudden death compared to the general population (Ficker et al., 1998). Furthermore, a number of other risk factors have been reported including high seizure frequency, seizure clusters, occurrence of generalised tonic-clonic (GTC) seizures, male sex, young age, subtherapeutic concentrations of AEDs, early onset of epilepsy, long duration of epilepsy, and treatment with three or more AEDs (Leestma et al., 1989; Shorvon, 1997; Kloster & Engelskjon, 1999; Nilsson et al., 1999; Walczak et al., 2001; Opeskin & Berkovic, 2003; Monte et al., 2007). However, no common risk factor has been found in all SUDEP cases.

At present the pathophysiology of SUDEP is unknown, although impaired autonomic regulation and seizure-induced cardiac changes have been implicated in its cause (Jansen & Lagae, 2010). Potential mechanisms of SUDEP include cardiac arrhythmias, autonomic imbalance, hypoxia, arrhythmogenic drugs and apnoea (Stollerberger & Finsterer, 2004). Neuropathological changes such as decreased brain weights, cerebral oedema and structural lesions have been reported in 27 to 70% of cases (Kloster & Engelskjon, 1999b; Garcia et al., 2001; Langan et al., 2005). Alterations in respiratory function have been reported during seizures, including tachypnoea, bradypnoea, apnoea and hypoxaemia (O’Regan and Brown, 2005). Pulmonary oedema and respiratory depression leading to apnoea, are commonly reported during seizures in human and animal studies which may be implicated in SUDEP and seizure-induced cardiomyopathy (Johnston et al., 1995; Johnston et al., 1997; Kloster & Engelskjon, 1999; Langan et al., 2005; Ryvlin, 2006).
Cardiac abnormalities, including post-mortem reports of myocardial ischaemia, seizure-induced QT-lengthening and interictal changes in heart rate variability have been described in patients with epilepsy (Ryvlin, 2006). Repetitive seizure-induced sympathetic dominance can lead to structural heart damage, increasing susceptibility to cardiac arrhythmias or ischaemia (Kloster & Engelskjon, 1999; Jansen & Lagae, 2010). Ion channel malfunctioning leading to ECG arrhythmias can be hard to identify during an autopsy and therefore cannot be ruled out as a contributor to SUDEP (Leung et al., 2006). ECG abnormalities can lead to arrhythmias, such as ictal ventricular tachyarrhythmias and ictal bradycardia/asystole (Schuele et al., 2007). Both of these have the ability to result in sudden cardiac death due to cerebral hypoperfusion and global hypoxia. Nei et al. (2004) reported that 94% of SUDEP patients had sinus tachycardia during or shortly after seizures, with a mean maximum HR of 149 b.p.m. Hearts from SUDEP patients are generally dilated and heavier than controls with non-fatal pathological changes reported in 33% of cases (Kloster & Engelskjon, 1999; Stollerberger & Finsterer, 2004). Post-mortem reports indicate the presence of fibrosis of the atrioventricular bundle or diffusely located in the myocardium (Kloster & Engelskjon, 1999). The pathological evidence suggests that SUDEP is caused by autonomic dysfunction leading to structural heart damage, as well as cerebral and respiratory oedema, thereby predisposing patients to sudden cardiac death.

1.3.8. Status epilepticus

The causes of SE are generally classified as acute versus chronic and require different management, respond differently and have varied outcomes. These acute processes include metabolic disturbances, central nervous system (CNS) infection, stroke, head trauma, drug toxicity and hypoxia (Lowenstein & Alldredge, 1998). Acute causes of SE are often difficult to control and are associated with a high mortality (Towne et al., 1994; Logroscino et al., 2005). In 12-30% of adults the occurrence of SE is the first episode of seizure before developing chronic epilepsy. Chronic processes generally refer to cases of pre-existing epilepsy where SE is due to a breakthrough of seizures or remote processes, such as CNS tumours or stroke and these patients generally respond well to antiepileptic therapy (Lowenstein & Alldredge, 1998).

Haemodynamic changes have been reported in patients following SE with deterioration of cardiac output due to decreased HR and mean arterial pressure causing gradual cardiac decompensation and myocardial injury (Boggs et al., 1998). Ten percent of patients who present with SE have myocardial infarctions based on their ECGs and confirmed by cardiac enzymes resulting in an overall mortality of up to 37% (Boggs et al., 1993; Boggs et al., 1998). Death due to SE is primarily caused by arrhythmias and global decompensation resulting from excessive
endogenous adrenaline release (Manno et al., 2005). The proposed mechanism of death following SE is most likely due to profound bradycardia or tachyarrhythmia during massive parasympathetic and sympathetic outflow coupled with mechanical weakness and respiratory distress (Sakamoto et al., 2008; Hotta et al., 2009; Hotta et al., 2010).

1.4. Kainic Acid Induced Excitotoxicity

1.4.1 Glutamate and glutamate receptors

Glutamate is the main excitatory neurotransmitter in the CNS which exerts its effects via activation of ionotropic and metabotropic receptors. Ionotropic glutamate receptors mediate fast neurotransmission and are subdivided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors (Bettler & Mulle, 1995; Castillo et al., 1997). NMDA receptors are voltage activated and non-selectively permeable to cations. The channel is blocked by Mg\(^{2+}\) ions which allows for a voltage dependent flow of K\(^{+}\) out of cell and an influx of Ca\(^{2+}\) and Na\(^{+}\) ions into the cell (McNamara, 1994). AMPA receptors are tetrameric cation channels made up of GluR\(_{1-4}\) subunits and are highly expressed throughout the CNS (Bettler & Mulle, 1995). The configuration of these subunits determines the permeability of the channel. A majority of AMPA receptors are impermeable to Ca\(^{2+}\) due to the presence of a GluR2 subunit, however AMPA receptors lacking the GluR2 subunit are highly permeable to Ca\(^{2+}\) and show fast desensitisation (Hestrin, 1993; Livsey et al., 1993; Bochet et al., 1994; Bettler & Mulle, 1995; Bleakman & Lodge, 1998). Kainate receptors are abundantly expressed in various brain regions, including the cerebellum, amygdala, hippocampus and spinal cord (Bettler & Mulle, 1995; Bleakman, 1999). They are involved in synaptic transmission, influencing both neuronal excitability and information transfer in the brain (Lerma, 2006). Kainate receptors are hetero- and homomeric ligand gated Na\(^{+}\) channels composed of GluR\(_{5-7}\) and KA\(_{1-2}\) subunits (Bleakman, 1999; Hollmann & Heinemann, 1994). Kainate receptors display rapid onset activation and desensitisation, and have a modulatory role at the synapse by presynaptically controlling neurotransmitter release, particularly in the hippocampus (Dakshinamurti et al., 1991; Bettler & Mullet, 1995; Bleakman, 1999; Jin et al., 2000; Lerma, 2006).

1.4.2. Glutamatergic excitotoxicity

Glutamate induced excitotoxicity is a mechanism of cell death in many neurological disorders including ischaemia, brain/spinal cord injuries, epilepsy, Alzheimer’s disease and Parkinson’s disease (Bettler and Mullet, 1995; Chen et al., 2002; Gleeson et al., 2010). Excitotoxicity is
triggered by the enhanced activation of metabotropic and ionotropic glutamate receptors (Lerma, 2009). Activation of AMPA and kainate receptors produces a robust influx of Na\(^+\) which strongly depolarises the cell, triggering the opening of NMDA and voltage gated Ca\(^{2+}\) channels (VGCC; Figure 1.9). This large influx of Ca\(^{2+}\) ions initiates many biochemical reactions, including reactive oxygen species production and activation of Ca\(^{2+}\)-dependent proteases, protein kinases, phospholipases and nucleases leading to mitochondrial dysfunction and apoptosis (Bettler and Mullet, 1995; Wang et al., 2005). Enhanced inflammatory responses, hypertrophy of astrocytes and microglia-macrophage activation also occurs which can result in further brain damage (Wang et al., 2005; Epsztein et al., 2009). Changes in electrolyte levels may also lead to neuronal cell death due to osmotic stress and cell lysis (Simon, 2009).

![Figure 1.9. Cellular mechanism of neuronal excitotoxicity following sustained glutamatergic stimulation. VGCC: voltage gated calcium channel, VGSC: voltage gated sodium channels.](image)
1.4.3. **Kainic Acid Induced Seizures**

Kainic acid (KA; 2-carboxy-4-isopropenylpyrrolidin-3-ylacetic acid; Figure 1.10) is a neuroexcitant derived from the red alga *Diginea simplex* (Coyle, 1987; Jane et al., 2009). It is a rigid analogue of L-glutamate which binds to AMPA and kainate receptors (Bleakman & Lodge, 1998). KA is 30-fold more neurotoxic than glutamate and has 5 to 30 times higher affinity for kainate receptors than AMPA receptors (Bleakman & Lodge, 1998; Shero et al., 1998; Wang et al., 2005; Lerma, 2006; Jane et al., 2009). Systemic administration of KA is used as an animal model of TLE, as seizures are initiated in the hippocampus before progressing to generalised epileptiform activity (Lothman & Collins, 1981; Ben-Ari, 1985; Wisden & Seeburg, 1993; Dernovsek & Sket, 1998; Epsztein et al., 2009). The most sensitive brain regions to KA induced excitotoxicity are the hippocampal CA1 and CA3 subregions and the hilus of dentate gyrus (Figure 1.11; Wang et al., 2005). These areas are rich in high-affinity kainate receptors and Ca\(^{2+}\) permeable AMPA receptors (Brorson et al., 1997; Lerma, 2009). KA induces an escalating level of seizure behaviours which include freezing, tremors, wet dog shakes and eventually clonic tonic convulsions (Goulton et al., 2010). This is a reliable model which has previously been used in our laboratory with great efficacy and reproducibility (Goulton et al., 2010; Read et al., 2014).

![Figure 1.10. Chemical structure of domoic acid, kainic acid and glutamate, agonists of glutamatergic receptors.](image)

![Figure 1.11. Histomicrograph of rat hippocampal regions stained with haematoxylin. CA: Cornu Ammonis areas, DG: dentate gyrus.](image)
1.5. Other seizure models

1.5.1 Domoic Acid

Domoic acid, like KA, is a potent agonist for AMPA and kainate receptors which induces wet dog shakes, freezing, tremors and eventually tonic-clonic convulsions in rats. Domoic acid causes a dose-dependent increase in seizure behaviours and can cause death from SE at high doses (Hesp et al., 2007; Sawant et al., 2010). Previous work in our lab demonstrated that at high doses, domoic acid (0.05-0.25 μM) and KA (0.5-2 μM) produced dose-dependent impairment of mitochondrial electron transport chain complexes I-V when administered directly to cardiac mitochondria (Vranyac-Trimoundanas et al., 2008). Interestingly, when domoic acid (0.05-10 μM) was administered to intact H9c2 rat cardiac myoblasts it did not compromise cellular viability (no change in cell quantification, lactate dehydrogenase leakage or reactive oxygen species). This lack of damage to intact cardiomyocytes raises further questions about the toxicological effects of domoic acid on the heart. These authors also examined the effect of domoic acid following systemic (2 mg/kg, ip) or intrahippocampal (100 pmol) administration (Vranyac-Trimoundanas et al., 2011). Seizure scores were similar between groups, although there was a time dependent decrease in cardiac haemodynamics (coronary flow rate, HR and left ventricular developed pressure). The cardiac haemodynamics and myopathy did not differ between the groups, suggesting that the cardiac damage observed is a consequence of seizure-induced cardiomyopathy rather than domoic acid acting directly on the myocardium.

1.5.2. Pilocarpine and Lithium

Pilocarpine is a non-selective muscarinic receptor agonist which produces prolonged, recurring seizures and brain damage via M1 receptor activation (Norell & Granstrom, 1980; Clifford et al., 1987; Berkeley et al., 2002). Lithium is often co-administered during pilocarpine-induced seizures as it improves the reliability of seizure induction in animal models (Clifford et al., 1987; Peredery et al., 2000). Lithium increases the pro-convulsant effect of pilocarpine by decreasing noradrenaline and dopamine release and enhancing ACh release (Haas & Ryall, 1977; Jope, 1979; Hruska et al., 1984; Clifford et al., 1985; Clifford et al., 1987). Within five minutes of pilocarpine administration, animals exhibit signs of peripheral cholinergic stimulation such as piloerection, salivation, tremor and diarrhoea before progressing to blinking, head bobbing, bilateral forelimb clonus and rearing (Honchar et al., 1983; Jope et al., 1986; Clifford et al., 1987). Animals develop SE within 25 minutes post-seizure induction and this may persist for 6 hours with death generally occurring in all animals within 24 hours (Jope et al., 1986; Clifford et al., 1987). To
reduce the peripheral side effects of pilocarpine, this model is often used with co-administration of a non-selective muscarinic antagonist, such as methscopolamine (Clifford et al., 1987), which may amplify cardiac dysfunction as a consequence of seizure. This model is very successful at producing reproducible SE and is associated with cardiac damage. However, pilocarpine seizures have a high mortality rate, and is not a good model for studying cardiac function due to the widespread parasympathetic activation.

1.5.3. Pentylentetrazol

Pentylentetrazol (PTZ) is a stimulant of the CNS with proconvulsant effects mediated by GABA\A antagonism (Nicoll & Padjen, 1976; Okada et al., 1989; Corda et al., 1992). PTZ also produces increased excitability by enhancing the permeability of potassium channels which decreases the recovery time between action potentials resulting in hyperexcitability (Madeja et al., 1996). PTZ can be used to produce an acute SE response or recurrent seizure activity by administering multiple sub-convulsant levels (Corda et al., 1991; Corda et al., 1992). This model is characterised by loss of postural control associated with tonic seizure and long-lasting uncontrolled clonic movements of all limbs. It is also associated with a mortality rate of up to 20%, with animals dying due to respiratory arrest (Corda et al., 1991; Velsek et al., 1992; Andre et al., 1998).

1.5.4 Electrical stimulation

Electrical stimulation can be used to examine the effect of seizures without exogenous chemical influences, and involves stereotaxically implanting electrodes into the required brain region. The most common sites include the amygdala, dorsal hippocampus, olfactory bulb and perirhinal cortex. A seizure is induced by high frequency trains of electrical stimulation (e.g. 30-100 Hz; McIntyre & Edson, 1987; McIntyre et al., 1987; Sarkisian, 2001; McIntyre, 2006; Sharma et al., 2007). This stimulation can be used acutely to produce neuronal epileptiform activity or to induce spontaneous seizure activity via kindling (repeat electrical stimulation; Sharma et al., 2007). Electrically induced seizures show greater specificity with less systemic side effects compared to chemoconvulsant agents (Sarkisian, 2001).

1.5.5. Genetic models

There are some well-established genetic models of spontaneous seizures in rats. The Wistar Albino Glaxo strain (WAG/Rij) rats are used as a model of absence epilepsy (Coenen et al., 1992). WAG/Rij rats show spike-wave discharges on the cortical EEG with discharges at 7-11
Hz for a duration of 1-45 seconds (Coenen et al., 1992). Other models include Strasbourg (GAERS) and Stargazer mice which present with absence seizures, or genetically epilepsy prone rats (GEPRs) which exhibit generalised tonic-clonic seizures (Holmes, 2004). These genetic models may induce seizure spontaneously or require activation, such as in audiogenic seizures. A disadvantage of these genetic models is that the channelopathies required to produce seizures can also be associated with cardiac modulation, therefore cardiac dysfunction has been reported in the absence of seizure activity (Damasceno et al., 2013).

1.6. Hypothesis and Aims

Aim 1
We propose that seizure activity results in acute cardiac dysfunction, producing tachycardia and ECG abnormalities (Figure 1.12). We hypothesise that sustained tachycardia, consequent to seizures, will produce arrhythmias and structural cardiac damage, which may be implicated in seizure-induced cardiomyopathy (Figure 1.12). Although ictal bradycardia is rare, occurring in only 2% of seizures, it cannot be dismissed as a contributor to sudden death in epilepsy as it may result in fatal bradyarrhythmias and asystole. The aim of the study reported in Chapter 2, is to investigate the acute effect of KA-induced seizure (10mg/kg, sc) on ECG activity over 180 minutes and to assess cardiac structure at 48 hours.

Aim 2
We propose that the cardiac dysfunction reported during seizures is due to altered autonomic function (Figure 1.12). Autonomic control of cardiac function can be assessed non-invasively though the use of heart rate variability (HRV). The second aim of this study (investigated in Chapter 3) is to evaluate the use of HRV, through pharmacological modulation, and to determine if seizure activity alters autonomic function in rats.

Aim 3
We hypothesise that seizure activity results in a “sympathetic storm” producing sustained tachycardia. Sustained or recurrent tachycardia can produce cardiac ischaemic injury which results in the development of micro-lesions and an increased risk of arrhythmias (Figure 1.12). The third aim of this study, which is reported in Chapter 4, is to investigate whether prophylactic therapy with the sympatholytics, atenolol and clonidine, will protect the heart during seizures.
Figure 1.12. Proposed mechanisms of seizure-induced cardiac dysfunction following acute or chronic seizure activity.

**Aim 4**
Systemic administration of KA produces a reliable and reproducible model of status epilepticus. Part four of this study (Chapter 5) assesses an intrahippocampal model of KA-induced seizure activity and further examines the effect of the sympathetic system in seizure-induced cardiomyopathy.

**Aim 5**
The fifth aim of the present study, investigated in Chapter 6, is to determine the benefit of interventional treatment with the antiepileptic drug, diazepam, in combination with the cardioprotective agent atenolol. It is hypothesised that blockade of seizure activity with diazepam will reduce seizure-induced cardiomyopathy, while atenolol is proposed to protect against sympathetically induced cardiac damage. It is also hypothesised that combination therapy (diazepam + atenolol) will provide superior benefit, by protecting both the heart and brain during seizure activity.
Chapter 2

Cardiac dysfunction following subcutaneous kainic acid-induced seizures
2.1 Introduction

Seizure activity has been increasingly associated with changes in autonomic function, particularly on the cardiorespiratory system, leading to cardiac arrhythmias, apnoea and hypoxia (Jansen & Lagae, 2010). Symptoms of increased autonomic activation frequently occur during seizures, with tachycardia (>100 b.p.m.) reported in the majority of clinical presentations (Provini et al., 1999; Opherk et al., 2002; Surges et al., 2009b; Jansen & Lagae, 2010). Electrocardiogram (ECG) wave abnormalities have been reported in 35% of seizures with potentially fatal changes occurring in 6-13% (Nei et al., 2000; Opherk et al., 2002; Devinsky, 2004). Clinically, it is important that these cardiac abnormalities are attenuated, particularly changes associated with risk factors of sudden cardiac death, such as tachycardia and QTc prolongation (Zipes & Wellens, 1998).

Within the different seizure classifications, status epilepticus (SE) has the highest mortality risk, with death generally occurring within 30 days of the initial convulsant activity (Towne et al., 1994; Logroscino et al., 2005; Metcalf et al., 2009a). Death often occurs in the absence of seizures and is believed to be due to an imbalance in autonomic function which results in altered cardiac control, electrical instability and increased risk of lethal cardiac arrhythmias (Metcalf et al., 2009b; Nguyen-Michel et al., 2014). Death following SE may result as a consequence of uncontrolled tachycardia leading to malignant ventricular tachyarrhythmias and fibrillation (Nei et al., 2000; Jansen & Lagae, 2010). Tachycardia is most commonly reported following generalised tonic-clonic convulsions and seizures of temporal origin (Terndrup et al., 1994; Metcalf et al., 2009a; Jansen & Lagae, 2010). Tachycardia increases the oxygen demand of the heart and can reduce coronary blood flow, thereby predisposing the ventricular tissue to ischaemic damage. This ischaemic injury can be further potentiated by the development of catecholamine-induced coronary vasospasm (Lyon et al., 2008). This ischaemic damage produces micro-lesions, increasing the risk of lethal arrhythmias and contributing to the deterioration of cardiac function (Boggs et al., 1993).

Ictal bradycardia (<40 b.p.m.) is rarely reported, occurring in fewer than 2% of seizures (Nei et al., 2000; Leutmezer et al., 2003; Devinsky, 2004) and is more prevalent in seizures of temporal or left sided origin (Devinsky, 2004). Bradycardia leading to asystole lasting more than 10 seconds, may impose severe systemic and cerebral hypoxemia (Surges et al., 2009b; Nguyen-Michel et al., 2014) and therefore should not be dismissed as a contributor to seizure related sudden death.
Systemic administration of KA in the rat, is widely used as an animal model of temporal lobe epilepsy (Goulton et al., 2010; Read et al., 2014). Seizures are initiated in the hippocampus, evoking focalised low level seizure behaviours, such as tremors and salivation (Goulton et al., 2010; Sawant et al., 2010; Read et al., 2014). As generalised seizures develop, animals display escalating behavioural responses such as wet dog shakes, forelimb clonus and eventually tonic-clonic convulsions (Dernovsek & Sket, 1998; Sawant et al., 2010).

Previous work in our laboratory has established that seizures induced by systemic or intrahippocampal administration of domoic acid, an analogue of KA, results in time dependent decreases in cardiac haemodynamics and function (Vranyac-Tramoundanas et al., 2011). This chapter investigates the effect of KA-induced seizures on ECG and EEG activity using indwelling telemetric devices in conscious rats and examines the extent of seizure-induced cardiomyopathy at 48 hours.
2.2 Methods

2.2.1 Materials: All reagents were purchased from BDH (Palmerston North, New Zealand) and Sigma-Aldrich (Auckland, New Zealand). KA (Tocris, Bristol, UK) was dissolved in normal saline. Surgical materials and animal remedies were obtained from the University of Otago Animal Welfare Office.

2.2.2 Animals: Sprague-Dawley rats (16 males; 330-350g) were obtained from the University of Otago Animal Resource Unit. The animals were housed on a 12 hour light/dark cycle at 22°C with food and water ad libitum and left to acclimatise for 5 days prior to surgery. Experiments were performed in accordance with the regulations of the University of Otago’s Committee on Ethics in the Care and Use of Laboratory Animals and the “Use of Laboratory Animals (NIH Publication No. 85-23, 1996)”.

2.2.3 Experimental Protocol: All animals (n=8/group) were implanted with a two channel transmitter with EEG and ECG electrodes, to allow simultaneous telemetric recordings of cortical and cardiac responses during seizures, as detailed below. Animals were randomised into control (no seizure induction) or seizure (KA administration) groups.

2.2.4 Surgical Implantation of Telemetric Transmitters: All surgical equipment was sterilised with hibitane (95% ethanol/5% chlorhexidine acetate). Animals were administered the antibiotic, amphioprim (0.2 mL, 60 mg/ml, bid), and the non-steroidal anti-inflammatory agent, carprofen (5 mg/kg, sc), 30 minutes prior to surgery and post-operatively for 3 days. Anaesthesia was elicited using ketamine hydrochloride (75 mg/kg, sc) and domitor (medetomidine hydrochloride; 0.5 mg/kg, sc) with atropine (0.05 mg/kg, sc). Body temperature was maintained at 37°C throughout the surgery using a warming blanket (Kent Scientific). Transmitter implantation and electrode positioning procedures were performed as previously described (Goultton et al., 2010; Sawant et al., 2010; Read et al., 2014).

The abdomen, neck and scalp of the animal was shaved and sterilised with hibitane prior to surgery to reduce risk of infection. A surgical drape was placed over the animal exposing only incision sites. When pedal withdrawal reflex was abolished, a 3 cm incision was made in the skin overlying the lower abdomen in line with the xiphoid process. A two-channel digital transmitter (Telemetry Research, Auckland, New Zealand, 35 x 25 x 10mm, 16 g) was inserted within a subcutaneous pocket on the lower abdomen.
The reference ECG electrode was sutured to the dorsal surface of the xiphoid process using a non-dissolvable 4.0 silk suture (Ethicon, USA). The recording ECG electrode was tunneled subcutaneously to the rostral thorax and placed into the anterior mediastinum to position the electrode close to the right atrium. The EEG electrodes were tunneled subcutaneously posterior to the foramen magnum. The abdomen was closed using a subcutaneous running suture (5-0 prolene suture, Ethicon, USA).

The animal was then positioned into a stereotaxic frame (David Kopf Instruments, USA), and an incision was made lengthwise along the scalp to allow retraction of the overlying skin. Bregma was used as the landmark for electrode placement. Three holes were drilled into the skull at a depth of 1 mm. The reference electrode was positioned 5 mm anterior to Bregma and 5 mm right of the midline and was secured at a depth of 1 mm from the skull surface using a stainless steel anchoring screw. The recording electrode was sited 5 mm posterior of Bregma and 5 mm left of the midline and inserted into the cortex at a depth of 2 mm from the top of the skull. The third hole allowed an anchoring screw to be placed 5.2 mm posterior of Bregma and 5 mm right of the midline and served to secure the electrodes to the skull using cranioplast cement to prevent displacement during seizure and the incision closed. Animals were housed individually post-surgery and left to recover for 7 days before seizure induction.

2.2.5 Seizure induction and Telemetric/Behavioural Recordings. Rat behaviour was observed in a custom-made perspex chamber (1m x 50cm x 50cm), in which each rat was left to acclimatise for 30 minutes prior to study initiation. EEG and ECG were sampled at 2000 Hz, with receiver filters set to 0.1 Hz high pass and 1000 Hz low pass using a Powerlab 2/25 signal conditioner and LabChart v.6 Pro software (AD Instruments, Sydney, Australia). EEG, ECG and behavioural data were simultaneously recorded during a 20 minute baseline period and for 3 hours post-saline or -KA administration, with periodic recordings taken at 24 and 48 hours. Seizures were induced by a single injection of KA (10 mg/kg, sc, maximum volume 0.35 mL), with control animals treated with an equivalent volume of saline subcutaneously. Following treatment, rats were immediately returned to the chamber for behavioural observation. Behaviour was recorded every 15 seconds for 3 hours with discrete changes in behavioural state additionally reported as they occurred. Behaviours were recorded using a 5-point scale as previously described (Table 2.2.1; Hesp et al., 2007; Sawant et al., 2010), with normal behaviours defined as Level 0, discomfort behaviours (Level 1), seizure behaviours confined to the head (Level 2), seizure behaviours associated with limbs or trunk such as wet dog shakes (WDS; Level 3), generalised seizure behaviours (Level 4) and tonic-clonic convulsion (Level 5). The cumulative score over the 180
The minute recording period was determined as the maximum score every minute. The mean behavioural score every 5 minutes was also analysed per rat. Arterial systolic blood pressure was recorded in a sub-group (n=4) of rats using a blood pressure tail cuff (ADInstruments, New Zealand), prior to seizure induction and at 15, 30, 60 and 180 minutes post-KA. Four repeat blood pressure recordings were taken using LabChart and the mean of the systolic blood pressure values determined.

2.2.6 EEG Analysis. EEG data was analysed using Fast Fourier transformation (FFT) to quantify the frequency bands: delta (1.25–4.50 Hz), theta (4.75–6.75 Hz), alpha (7.00–12.50 Hz), beta (12.75–35 Hz), and gamma (35.5–100 Hz) over 5 minute bins. These sampling periods were analysed by dividing them into half overlapping half-second epochs, using a weighted Cosine bell window (Lab Chart Pro, Spectral Analysis). Power spectral density (PSD) for each period was calculated as the sum of all epochs. Baseline PSD for each animal was determined during acclimatisation and all PSD values were expressed as a percentage of baseline.

<table>
<thead>
<tr>
<th>Table 2.2.1. Behavioural scores allocated to observed rat behaviours</th>
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<tr>
<td><strong>Level 0:</strong> Normal Behaviours</td>
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<tr>
<td><strong>Level 1:</strong> Discomfort behaviours</td>
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<tr>
<td><strong>Level 2:</strong> Mild seizures, stereotypical behaviours confined to the head and neck region</td>
</tr>
<tr>
<td><strong>Level 3:</strong> Moderate seizure, stereotypical movements of the limbs or trunk</td>
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<tr>
<td><strong>Level 4:</strong> Severe generalised seizure behaviours</td>
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2.2.7 ECG Analysis. ECG data was analysed using LabChart6 Pro ECG Analysis module software in order to assess heart rate (HR), QT intervals and T wave amplitudes. Data were analysed every two minutes in one minute blocks over the 3 hour observation period. HR increased inversely as the RR interval decreased, resulting in a reduced QT interval. The converse was also true as HR decreased. Therefore, the corrected QT (QTc) was calculated in order to adjust for the rate by applying the Mitchell algorithm to the QT interval recordings,
Chapter 2: Cardiac dysfunction following subcutaneous KA-induced seizures

where \[ QT_c = \frac{QT_0}{(RR/100)^{1/2}} \] (Mitchell et al., 1998). This algorithm is designed to correct for the higher HR and altered QRS-T wave morphology in rodents.

2.2.8 Morphological Characterisation of Myocardial Injury: 48 hours following KA or saline administration animals were anaesthetised with halothane and the heart excised. Hearts were arrested in diastole by flushing with 50 ml of ice-cold (4°C) 0.9% saline containing 20 mM of KCl. The tissue was perfusion-fixed (73.6 mmHg pressure) and maintained in 10% neutral buffered formalin (NBF; LabServ, Thermo Fisher, New Zealand) for 12 hours and stored in 70% ethanol. Apical ventricular tissue (6 mm depth from apex) was paraffin-embedded and tissue sections (5 μm) prepared for staining with Martius scarlet blue (MSB) trichrome stain for collagen localisation. Tissue processing and wax embedding were carried out by the Histology Service Unit, Department of Pathology, University of Otago, NZ. Tissues were dehydrated with ethanol (70-100%), before immersion in 65°C paraffin, then paraffin under vacuum. Processed tissue was set into paraffin blocks, before sectioning on a microtome at 3 μm, and mounting on slides. Slides were dried at 60°C for 1 hour and stored at room temperature until staining. MSB staining involved emerging slides in martius yellow for 2 minutes, followed by crystal scarlet for 10 minutes. Slides were treated with phosphotungstic acid until only fibrin was red (up to 10 minutes). Qualitative histological evaluations were randomly performed in a blind manner, on 10 individual digital images from each section (30 total, 20x magnification), using an Axioplan-2 microscope and recorded with the Axiovision v.3.1 image analysis system (Carl Zeiss Ltd., Germany). Semi-quantitative analysis was conducted using Adobe Photoshop CS5 software in which the ratio of collagen stained (blue) or oedema (white) pixels were calculated against background in each cardiac section.

2.2.9 Statistics. Statistical analysis was performed using PrismTM v.5 (GraphPad, San Diego, USA). Behavioural data were analysed using a Kruskal-Wallis test with Bonferroni post-hoc. ECG variables were analysed using a 2-way repeated measures AVOVA with Bonferroni post-hoc analysis. Statistical significance was determined as \( P < 0.05 \). Data are presented as mean ± standard error of the mean (SEM).
Chapter 2: Cardiac dysfunction following subcutaneous KA-induced seizures

2.3 Results

2.3.1 Seizure Activity

Behavioural and power spectrum density (PSD) remained constant in the control group during the 180 minute behavioural study (Figure 2.3.1), where animals displayed exploring, grooming and resting behaviours. In the control group, $2 \pm 0.2$ WDS behaviours were observed while no other Level 3 or 4 behaviours occurred (data not shown). Administration of KA immediately increased behaviour scores ($P<0.05$, Figure 2.3.1B) with a cumulative score of $463 \pm 30$ recorded over the 180 minute period (compared to $7 \pm 2$ in the control group). The progressive increase in behavioural score following KA, corresponded to an increase in EEG activity (Figure 2.3.1). During the initial 30 minutes following KA there was a significant increase in the theta (4.75-6.75 Hz) frequency band ($P<0.05$, Figure 2.3.1) corresponding to Level 1 and 2 seizure behaviours. As seizure severity increased, the power across all frequency bands became elevated by 5 to 21-fold ($P<0.05$ compared to baseline). All behavioural and EEG activity had returned to baseline by the 24 hour and 48 hour recording periods in the seizure group (data not shown).

2.2.2 Cardiac Function

The mean baseline HR for the Control group was $352.9 \pm 15.8$ b.p.m. which did not significantly change during the study (Figure 2.3.2). KA administration caused a maximal decrease in HR by 28.1% (to $272.6 \pm 32.9$) 10-18 minutes post-KA ($P<0.05$). During this bradycardic period all animals had evidence of bradyarrhythmias, with premature ventricular contraction (in 7/8 animals) and AV block (in 7/8 animals) commonly observed (Figure 2.3.5). As seizure severity increased, there was an increase in HR by 13-28% at 60-124 minutes post-KA with a maximum mean HR of $482.7 \pm 12.2$ b.p.m. at 74 minutes ($P<0.05$, Figure 2.3.2). The baseline QTc interval for the Control and KA groups were $42.4 \pm 2.0$ msec and $44.3 \pm 1.4$ msec, respectively. Administration of KA resulted in prolongation of the QTc interval by 25-38% over 50-180 minutes. The QTc interval remained prolonged by 30% at the 24 hour recording period ($P<0.05$, Figure 2.3.3) but returned to baseline by 48 hours.

Baseline T wave amplitudes ranged from $0.08-0.23$ mV in the control group and $0.16-0.31$ mV in the KA group. As T wave amplitude depends on electrode placement, amplitude was normalised to the mean baseline per rat. No significant change in the T wave amplitude was observed in the control group over the course of the study, while KA administration resulted in a progressive increase in the T wave amplitude by 47.8-98.4% over the 66-180 minute recording period ($P<0.05$, Figure 2.3.4). There was no significant change in BP during the bradycardic period but as tachycardia developed, BP increased by 26% at 1 hour (Figure 2.3.6).
Figure 2.3.1. Effect of saline (black bars) or KA (10 mg/kg, sc, white bars) on EEG (A) and behavioural (B) activity every 5 min over 180 min. EEG activity was presented as power spectrum density (PSD) of each frequency band: delta (1.25-4.5 Hz), theta (4.75-6.75 Hz), alpha (7.0-12.5 Hz), beta (12.75-35.0 Hz) and gamma (35.0-100 Hz). Values normalised to 30 min baseline recording and presented as mean ± SEM. *P<0.05 compared to control.

Figure 2.3.2. Effect of saline or KA (10 mg/kg, sc) on heart rate (beats per minute, b.p.m.) in rats. Data was analysed every 2 minutes for 1 minute over 180 min with a 30 min period at 24 and 48 hours and presented as mean ± SEM. *P<0.05 compared to control.
**Figure 2.3.3.** Effect of saline or KA (10 mg/kg, sc) on the QTc interval ($QTc = QT0/(RR/100)^{1/2}$) in rats. Data was analysed every 2 minutes for 1 minute over 180 min with a 30 min period at 24 and 48 hours and presented as mean ± SEM. *P<0.05 compared to control.

**Figure 2.3.4.** Effect of saline or KA (10 mg/kg, sc) on the T wave amplitude in rats. Data was analysed every 2 minutes for 1 minute over 180 min with a 30 min period at 24 and 48 hours. Data was normalised to the baseline T wave amplitude and presented as mean ± SEM. *P<0.05 compared to control.
Figure 2.3.5. Representative ECG traces during the 30 min following KA administration. Baseline, bradycardia, premature ventricular contraction (PVC) and atrioventricular (AV) block.

Figure 2.3.6. Effect of KA (10 mg/kg, sc) on heart rate (beats per minute, grey line) and systolic blood pressure (black line) in rats. Presented as mean ± SEM. #P<0.05 compared to baseline.
Chapter 2: Cardiac dysfunction following subcutaneous KA-induced seizures

Microscopical examination of the midplane sections of left ventricular myocardium showed evidence of sub-endocardial damage at 48 hours post-KA (Figure 2.3.7/8). Figure 2.3.7 clearly demonstrates the appearance of patches of fibrotic plaques throughout the myocardium of a KA-treated animal, including perivascular (Figure 2.3.7B) and interstitial (Figure 2.3.7C) fibrosis. Colour pixel counting of the MSB stained sections provided clear quantitative evidence of dense collagen deposition (fibrosis) encapsulating necrotic cells throughout the myocardium of KA treated animals ($P<0.05$, Figure 2.3.8D/E, Table 2.3.1). There was significant evidence of ventricular oedema (Figure 2.3.8C, Table 2.3.1) and myocyte vacuolisation with nuclear displacement, indicative of early reversible ischaemic damage (Figure 2.3.8C). Multifocal lesions evidenced by hypercontraction band necrosis and muscle fibre degeneration were also found in 62% of micrographs assessed (Figure 2.3.8E/F, Table 2.3.1). Non-specific inflammatory cell infiltration was observed in the sub-endocardial of all KA animals (Figure 2.3.8D/E).

**Table 2.3.1. Relative extent of morphological features 48 hours post-seizure onset in midplane left ventricular sections**

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<tr>
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<th>Control</th>
<th>Kainic Acid</th>
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<tr>
<td><strong>Fibrosis</strong></td>
<td>0.1 ± 0.03 %</td>
<td>0.9 ± 0.3 % *</td>
</tr>
<tr>
<td><strong>Oedema</strong></td>
<td>7.5 ± 4.2 %</td>
<td>30.7 ± 5.0 % *</td>
</tr>
<tr>
<td><strong>Myocyte Vacuolisation</strong></td>
<td>0.02 ± 0.02</td>
<td>6.2 ± 1.1 *</td>
</tr>
<tr>
<td><strong>Hypercontracture Band Necrosis</strong></td>
<td>0 ± 0</td>
<td>7.7 ± 1.1 *</td>
</tr>
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Quantification of each feature was conducted on 10 fields from each of the three ventricular sections. Fibrosis and oedema were quantified as the number of positively stained pixels per field. Myocyte vacuolisation and hypercontracture band necrosis were graded on the presence of 2+ vacuolised nuclei positive cells in each field.
Figure 2.3.7: Representative micrograph (A) of a ventricular section at 48 hours post KA administration. Heart stained with MSB, showing diffuse distribution of collagen deposition (blue). Smaller micrographs (B-C) demonstrating example micrograph images (20x) randomly assessed for fibrosis deposition.
Figure 2.3.8. Representative micrographs of left ventricular subendocardium collected at 48 hours following A/B) saline administration to control animals showing normal representative myocardium; (C-F) Effects of KA (10 mg/kg, sc) administration showing; C) myocyte vacuolisation (arrows) and oedema; D) coagulative myocytolysis illustrated by necrosis associated with inflammatory cell infiltration and interstitial fibrosis; E/F) hypercontracture band necrosis with fibrosis. Scale bar represents 100 µm.
2.4 Discussion

This study clearly shows that a high systemic dose of KA induces generalised seizure activity which develops to status epilepticus. This persistent seizure activity leads to prolonged ECG changes, arrhythmias and structural damage within 48 hours of the initial seizure event. Immediately following the KA injection, animals appeared hypoactive, by resting abnormally and belly pressing, before progressing to head tremor and WDS. This is similar to previous studies where animals exhibited staring behaviours and tremors before developing limbic seizures (Lothman & Collins, 1981; Chen et al., 2002; Goulton et al., 2010).

During the 40 minutes following KA administration, the EEG traces revealed an increase in the theta and alpha frequency bands associated with mild seizure behaviours. Activation of these lower frequencies are associated with neurological diseases, specifically temporal lobe epilepsy (Zaveri, 2001). These low seizure behaviours coincided with a bradycardic period where HR dropped by 28% and bradyarrhythmias developed in a majority of animals (Figure 2.4.1). Similar effects have been reported by Ferrari et al. (2008) where HR decreased by 22% within 30 minutes of KA (12 mg/kg, sc) administration. In this study, the reported bradycardia was not attenuated by adrenalectomy leading the authors to suggest that the change in HR was most likely due to enhanced parasympathetic activity rather than decreased sympathetic stimulation (Ferrari et al., 2008). This conclusion was supported by Sakamoto et al. (2008) where KA (10-12 mg/kg, ip or iv) administration in urethane anesthetised rats produced a sustained increase in vagal nerve activity (4.9-fold) with 41% of animals dying due to profound bradycardia or arrhythmia, such as AV block. Using the same model, however, Hotta et al (2009) reported a drop in HR caused by decreased cardiac sympathetic nerve activity in rats lacking ventilation. Amyloid kindling in rats, produced an immediate short-lasting drop in HR by ~50% which coincided to an elevation in BP (~35%) which was not replicated during bradycardia in the current study (Goodman et al., 1999).

In the current study, KA administration caused a progressive increase in EEG activity and seizure behaviours, such as myoclonic jerks and foaming, which coincided with the development of tachycardia, QTc prolongation and T wave elevation (Figure 2.3.9). The elevated sympathetic responses (tachycardia, hypertension and tachypnoea) observed in this study during generalised seizure activity supports previous animal and clinical studies (Terndrup et al., 1994; Metcalf et al., 2009a). In an anaesthetised rat model, Hotta et al reported a 17% increase in HR and a 2-fold increase in sympathetic nerve activity within an hour of KA (10-12 mg/kg, ip) administration.
(Hotta \textit{et al.}, 2009; Hotta \textit{et al.}, 2010). Metcalf \textit{et al} (2009a) and Bealer \textit{et al} (2010) showed that SE, induced by lithium and pilocarpine (with methyscolopine) in rats, caused significant increases in HR (~30-35%) and BP (~25-30%) within 60 minutes, which coincided with elevated troponin I levels, indicative of acute myocardial injury. The QTc interval (~30-40%) was also prolonged in these animals 10-14 days post-SE and was associated with an increased aconitine-induced arrhythmia risk. Naggar \textit{et al.} (2013) reported significantly elevated HR (33%), mean arterial pressure (19%) and QTc prolongation, attributed to decreased vagal tone, in a chronic (2-11 months) KA seizure model. Ohta \textit{et al.} (1991) reported a KA-induced dose-dependent (0.1-1 mg/kg KA, iv) increase in pressor and tachycardiac responses following by a prolonged period of bradycardia. These results, combined with prior literature, suggest sympathetic dominance during generalised seizure activity which may contribute to sudden cardiac death in epileptics (Naggar \textit{et al.}, 2014).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.4.1}
\caption{Representative time matched recordings of EEG (mV; grey spectral trace), heart rate (beats per min; black line trace) and behavioural responses in individual rats following saline (A) or KA (B, dashed line) administration.}
\end{figure}

This study clearly showed that 48 hours following a SE event there is a deterioration in cardiac morphology. The KA treated hearts showed evidence of nuclear vacuolisation, a marker of
reversible ischaemic damage, and hypercontraction band necrosis, suggestive of elevated catecholamine levels (Boggs et al., 1993; Kloster & Engelskjon, 1999; Manno et al., 2005; Tsang et al., 2008; Jansen & Lagae, 2010). Hypercontracture band necrosis was reported in 73% of patients who died due to SE which led the authors to suggest sympathetic involvement in SE-induced death (Manno et al., 2005). The pathology of the hearts in the present study, was similar to both animal and clinical studies (Natelson et al., 1998; Kloster & Engelskjon, 1999; Stollerberger & Finsterer, 2004; Metcalf et al., 2009a). Structural changes have been reported in 33% of epileptic patients with evidence of fibrosis, reversible myocyte vacuolisation, leukocytic infiltration and oedema found post-mortem (Natelson et al., 1998; Kloster & Engelskjon, 1999). The fibrotic deposition observed in the present study, resembled pathology reported by Pick et al. (1989) following isoprenaline (1 mg/kg) administration in a rat, suggesting that elevated catecholamine levels are implicated in seizure-induced cardiomyopathy.

Repeated hypoxaemic insults and increased catecholamine levels can cause structural heart damage, making the heart more susceptible to fatal arrhythmias. Haemodynamic changes have also been reported following SE, with deterioration of cardiac output due to decreased HR (8-40 b.p.m) and mean arterial pressure (15-52 mmHg). These patients presented with gradual cardiac decompensation and myocardial injury due to elevated endogenous catecholamine release (Boggs et al., 1998; Manno et al., 2005). Boggs et al. (1993) reported a mortality rate of 25% in SE patients, with 10% of these patients having myocardial infarction indicated by ECG and confirmed by cardiac enzymes.

This chapter clearly demonstrates that KA administration produces significant behavioural and EEG changes which coincide with alterations in cardiac electrographical activity, resulting in significant structural damage. These results suggest altered cardiac autonomic control during seizure activity, may contribute to the increased risk of fatal arrhythmias, such as asystole, or deterioration of cardiac function due to tachycardia-induced cardiomyopathy. This highlights the importance of initiating a therapy in epileptic patients to protect cardiac function and preserve morphology.
Chapter 3

Heart rate variability during subcutaneous kainic acid-induced seizures
3.1 Introduction

The autonomic system is important for controlling cardiovascular homeostasis though the balance of sympathetic and parasympathetic modulation. Seizure activity is associated with changes in autonomic function, as observed by altered HR, blood pressure, flushing, tachypnoea, salivation and sweating (Naritoku et al., 2003; Devinsky, 2004; Harnod et al., 2008; Jansen & Lagaë, 2010). Activation of the sympathetic system results in increased HR and force of contraction through activation of β adrenergic receptors, primarily β1 (Vaseghi and Shivkumar, 2008; Figure 1.3 and 1.4). Conversely, parasympathetic activation has negative ionotropic and chronotropic effects through vagal stimulation of the SA and AV node.

The beating of a healthy heart _in situ_ is not absolutely regular, as it undergoes fine beat-to-beat tuning. Mean HR fluctuates as a consequence of variations in circadian rhythms, rest, exercise and stress (Stein _et al._, 1994). In addition, the intervals between normal sinus beats can vary periodically under the influence of respiration rate, baroreflex, thermoregulation and renin-angiotensin-aldosterone system changes (Figure 1.6; Akselrod _et al._, 1985; Camm _et al._, 1996; Stauss, 2003). Sympathetic and vagal stimulation of the SA are characterised by discharge synchronised with each aspect of cardiac cycle. This ANS control is further modulated by central and peripheral systems, such as vasomotor and respiratory mechanisms, generating rhythmic oscillations in the heart rate period.

The resultant heart rate variability (HRV) caused by variations in the sympathovagal balance has been measured using either time domain or frequency domain analysis techniques (van Ravenswaaij _et al._, 1993; Naritoku _et al._, 2003; Acharya _et al._, 2006). Time domain analysis of HRV is a simple form of statistical calculations performed on a set of inter-beat intervals (Stein _et al._, 1994). It gives an indication of total variability or can be used to compare the lengths of adjacent cycles. Comparison of RR intervals which differ by greater than 50 ms is used as a measure of vagal activity in humans (Camm _et al._, 1996), however this measure is not applicable in the rat. The standard deviation of normal RR intervals (SDNN) can be used as a measure of overall variance in a recording, however variability increases with the length of the recording so it is important that the SDNN is measured over 0.5-5 min or 24 hours (Seely & Macklem, 2004).

Frequency domain analysis of the RR interval provides insight into the underlying rhythms affecting contraction. The total power reflects all cyclic components responsible for variability in the period of recording (Stein _et al._, 1994). Fourier analysis of the RR interval is used to divide the total variance into underlying groups of frequencies. The low frequency (LF, 0.04-0.15 Hz) band is associated with both sympathetic and parasympathetic modulation, as well as strongly
Chapter 3: Heart Rate Variability during KA-induced seizures

representative of the baroreflex (van Ravenswaaij et al., 1993; Stein et al, 1994). In comparison, the high frequency (HF, 0.15-0.04 Hz) band is controlled almost exclusively by parasympathetic effects and has a large respiratory component. Therefore, HRV analysis can provide a non-invasive indicator of cardiac autonomic control.

Epilepsy is associated with altered cardiac autonomic function which leads to changes in HRV (Table 1.2 from Chapter 1). Frequency analysis of patients with temporal lobe seizures suggests altered cardiac innervation with all studies reporting changes in power and reduced overall variability compared to controls, although there are inconsistencies as to how TLE affects the sympathovagal balance (Massetani et al., 1997; Tomson et al., 1998; Druschky et al., 2001; Ansakorpi et al., 2002; Ansakorpi et al., 2004; Dutsch et al., 2006; Ponnusamy et al., 2011; Dericioglu et al., 2013). Generalised tonic-clonic seizures, however, are commonly associated with sympathetic dominance (Evrengul et al., 2005; Dutsch et al., 2006; Hattori et al., 2007; Harnod et al., 2008; Harnod et al., 2009; Mativo et al., 2010).

This chapter aims to examine how systemic administration of KA alters the balance of the autonomic system, to produce an immediate period of bradycardia followed by elevated HR (as described in Chapter 2). Ipratropium, a hydrophilic non-selective muscarinic blocker, was used to block cardiac parasympathetic modulation, while atenolol was used to block β₁ mediated sympathetic activity. This chapter also examines HRV analysis in the rat and determines the acceptable parameters for frequency analysis. It is hypothesised that KA alters autonomic function due to seizure activity affecting specific brain regions, resulting in an immediate activation of the parasympathetic system followed by a sympathetic storm.
3.2 Methods

3.2.1. Material. All reagents were purchased from BDH (Palmerston North, New Zealand) and Sigma-Aldrich (Auckland, New Zealand). Prescription remedies were obtained from the Drug Control Officer at the University of Otago Animal Welfare Office. KA (Tocris), ipratropium (Sigma-Aldrich) and atenolol (Sigma-Aldrich) were dissolved in saline.

3.2.2. Animals. Sprague-Dawley rats (15 males; 320-350g) were obtained from the University of Otago Animal Resource Unit. The animals were housed on a 12 hour light/dark cycle at 22°C with food and water ad libitum and left to acclimatise for 5 days prior to surgery. Experiments were performed in accordance with the regulations of the University of Otago’s ethics committee.

3.2.3. Experimental protocol. All animals (n=5/group) were implanted with a two channel transmitter, as detailed in Chapter 2.2.4. Animals were randomised into saline, ipratropium or atenolol treated groups. ECG activity was recorded over a 30 minute pre-treatment period. Animals were then administered a bolus subcutaneous injection of saline, atenolol (5 mg/kg) (Oppenheimer & Cechetto, 1990; Zhang & Cheng, 2000), or ipratropium (5 mg/kg)(Leusch et al., 2001) and HR was recorded for a further 30 minutes (baseline) at which time KA (10 mg/kg, sc) was given to all animals. Behaviours and ECG were recorded for 3 hours following KA (9 am to 12 pm), at which time the rats were sacrificed.

3.2.4. Data Analysis. Behavioural data was expressed as the mean behavioural score every 10 minutes. ECG data was analysed using LabChart6 Pro ECG Analysis module software. Data were analysed every two minutes in one minute blocks over the 4 hour observation period. HRV was analysed every 30 minutes over a 5 minute period using the LabChart6 Pro HRV Analysis module software. Total power was determined by FFT analysis across all frequencies. Low frequency was determined as 0.04-0.5 Hz and high frequency as 0.5-3 Hz as determined by baseline spectral analysis conducted in all rats. Data was presented as the power within the each frequency band and normalised to the total power within the 0.04-3 Hz frequency band.

3.2.5. Statistics. Statistical analysis was performed using Prism™ v.5 (GraphPad, San Diego, USA). Behavioural data were compared using a Kruskal-Wallis test with Bonferroni post-hoc analysis. ECG variables were analysed using a 2-way repeated measures AVOVA with Bonferroni post-hoc analysis. Statistical significance was determined as $P<0.05$. Data are presented as mean ± standard error of the mean (SEM).
3.3 Results

3.3.1 Heart Rate Variability Analysis in the rat

3.3.1.1 Determining Frequency domain analysis in a rat

Physiological heart rate responses vary from beat to beat and fluctuates throughout the day. Figure 3.3.1 is an example of a rat’s average HR every minute over a 24 hour recording period. In this rat, HR varied substantially with a minimum HR of 274 b.p.m at 12:01 pm and a maximum of 436.4 b.p.m at 5:03 am. Spectral analysis was performed on all rats during baseline (n=15) and the peak LF and HF band was recorded. The mean LF and HF peaks were 0.13 ± 0.03 Hz (ranging from 0.039-0.48 Hz) and 1.41 ± 0.12 Hz (ranging from 0.55-2.34 Hz), respectively, resulting in the use of 0.04-0.5 Hz for the low frequency band and 0.5-3 Hz for the high frequency band (Figure 3.3.2).

![Graph showing heart rate variability](image)

**Figure 3.3.1.** The heart rate (beats per min, b.p.m.) in an untreated rat over 24 hours. The average HR every minute was recorded.
3.3.2.2 Effect of ipratropium and atenolol on frequency domain analysis

During the baseline period, the RR interval fluctuated from beat to beat (Figure 3.3.4 and 5) with the ipratropium and atenolol groups showing an average RR interval of 0.17 and 0.18 seconds, respectively, over a 5 minutes recording period. Both ipratropium and atenolol administration decreased RR interval variability (Figure 3.3.3 and 4). Ipratropium reduced the mean RR interval by 29% to 0.12 sec. This decrease corresponded to a drop in the LF and HF bands, confirming a reduction in parasympathetic activity. In comparison, atenolol administration increased the RR interval by 17% to 0.21 sec, which matched a reduction in the LF band power (Figure 3.3.4).
Figure 3.3. An example of individual RR intervals (s) over a 5 min (A) and 30 sec (B) recording period before and after ipratropium (5 mg/kg, sc) administration in a single rat. Panel C shows the spectral analysis of the 5 min recording period shown in A. LF = low frequency, a measure of sympathetic and parasympathetic activity. HF = high frequency, a measure of parasympathetic activity.
Figure 3.3.4. An example of individual RR intervals (s) over a 5 min (A) and 30 sec (B) recording period before and after atenolol (5 mg/kg, sc) administration in a single rat. Panel C shows the spectral analysis of the 5 min recording period shown in Panel A. LF = low frequency, a measure of sympathetic and parasympathetic activity. HF = high frequency, a measure of parasympathetic activity.
3.3.2 Pharmacological autonomic modulation

3.3.2.1 Behavioural

Administration of ipratropium or atenolol had no effect on seizure behaviours during the baseline period. KA administration resulted in the immediate development of seizure behaviours in all groups, with all saline treated animals presenting with Level 3 behaviours by 60 minutes (Figure 3.3.5A). Interestingly, a single bolus injection of atenolol prior to KA reduced seizure severity 110-180 minutes post-KA (Figure 3.3.5A), as well as significantly decreasing Level 4 and 5 behaviours by 56% and 92.6%, respectively (Figure 3.3.5D and E). Ipratropium did not affect seizure behaviour progression, however ipratropium treated animals had a significantly higher cumulative behavioural score (469.25 ± 36.34) compared to the atenolol treated animals (289.6 ± 41; Figure 3.3.5B).

![Graph showing behavioural scores over 180 min following KA administration](image)

**Figure 3.3.5.** Behavioural scores over 180 min following KA administration (dashed line) in rats. Pre-treatment behavioural score was taken for 30 min prior to saline, ipratropium or atenolol administration (solid line). A) Behaviour scores are presented as the mean over 10 min. Cumulative score represented the sum of the maximum behaviour every minute (B). The total number of wet dog shake (WDS; C), Level 4 (D) and Level 5 (E) behaviours over 180 min were also recorded. Data represents the mean ± SEM. *P<0.05 compared to saline, †P<0.05 compared to ipratropium.
3.3.2.2 Heart Rate

There was no significant difference in HR between groups during the pre-treatment period. In the saline pre-treated group, KA administration decreased HR by 20-34% during the initial 2-40 minutes. As seizure severity increased so did HR with an increase of 19-29% at 64-128 minutes (maximum HR of 504 ± 10 at 74 minutes; Figure 3.3.6A and B). Ipratropium administration resulted in an immediate and sustained increase in HR (Figure 3.3.6A). KA administration in the ipratropium group resulted in a maximum decrease in HR of 11% (396 ± 6.4 b.p.m.) at 22 minutes. Ipratropium pre-treatment also reduced the extent of tachycardia caused by KA, with a maximum increase in HR of 14% (HR of 510 b.p.m.) compared to baseline. Atenolol administration decreased HR to 302 ± 11 b.p.m. during the baseline period. Induction of seizures with KA resulted in a minimum HR of 225 b.p.m. which was maintained until 38 minutes. Significant increases (12-18%) in HR were recorded 78-112 minutes post-KA in the atenolol treated group, with a maximum HR of 357 ± 20 b.p.m. (Figure 3.3.6A and D).

![Figure 3.3.6. Effect of KA administration on heart rate (beats per min, b.p.m.) over 180 min (A). HR was normalised to the baseline phase (-30-0 min) to examine the effect of seizure following treatment with saline (B), ipratropium (C) or atenolol (D). Data represents the mean ± SEM. *P<0.05 compared to baseline, * P<0.05 compared to saline, +P<0.05 compared to ipratropium.](image-url)
3.3.2.3 Heart Rate Variability analysis following KA

HRV values were comparable between groups during the pre-treatment recording period. Figure 3.3.7 shows an example of spectral analysis following KA administration in a single rat. Seizure activity increased the variability of the RR interval (Figure 3.3.7A) throughout the 180 minute study. During the bradycardia phase (15-60 min), there was an increase in power across the spectral analysis, demonstrating uncontrolled cardiac modulation (Figure 3.3.7B). Spectral analysis started returning back to baseline at 120 minutes which coincided with a reduction in HR towards baseline values. In the saline group, KA administration did not significantly alter the SDNN, although there was a significant increase in total power by 147% at 15 minutes, respectively (Figure 3.3.8A and B). A bolus injection of KA resulted in altered autonomic activity as demonstrated by an increase in HF power by 7-fold to 45 ± 16 ms² which corresponded to a drop in HR by 25-30% (P<0.05; Figure 3.3.8E). The power of the LF band was also significantly increased by 1.8-fold 15 minutes following KA (Figure 3.3.8C). Expression of LF and HF as normalised units, clearly showed that seizure resulted in enhanced vagal activity at 15-90 minutes producing parasympathetic dominance at 30-60 minutes when the LF/HF ratio dropped by 55-62% (Figure 3.3.8D, F and G). As expected, when HR started to return back to baseline the LF/HF ratio also returned to normal levels.

Ipratropium administration significantly reduced overall HRV, seen as a reduction in the SDNN, TP and LF (P<0.05) which was further diminished during seizure activity (Figure 3.3.5A and B). HF power was also significantly decreased by 82% to 2.28 ± 1.3 ms² with ipratropium. This reduction was significantly enhanced during seizure activity where HF power dropped to 0.84 ± 0.2 ms² at 90 minutes. Surprisingly, pre-treatment with ipratropium decreased the LF/HF ratio by 54% to 0.68 ± 0.12 and this remained reduced following KA administration (Figure 3.3.8.G).

Atenolol had no significant effect on HRV parameters during pre-treatment. KA administration resulted in an increase in the total power which corresponded to an increase in the LF power by 4.5-fold and HF power by 11-fold at 15 minutes post-KA. Atenolol pre-treatment prevented the development of tachycardia (max HR of 357 ± 20 b.p.m.) due to a reduced LF/HF ratio 150 minutes post-KA (Figure 3.3.6D, 3.3.8.G).
Figure 3.3.7. Effect of seizures on heart rate variability. A) RR interval (seconds) following KA administration over 180 min. B) Spectral analysis over a 5 min recording period prior to KA and at 15, 30, 60, 120 and 180 min post KA.
Figure 3.3.8. The effect of seizures on heart rate variability. Standard deviation of normal RR intervals (SDNN, milliseconds; A), total power (ms², B), low frequency (LF, 0.04-0.5 Hz) analysis expressed as raw (ms², C) or normalised (nu: normalised unit; D), high frequency (HF, 0.5-3 Hz) analysis expressed as raw (ms², E) or normalised (nu: normalised unit; F) and frequency/high frequency ratio (LF/HF; G) following treatment with saline, ipratropium or atenolol prior to KA administration. Data represents the mean ± SEM. *P<0.05 compared to pre-treatment, †P<0.05 compared to baseline, *P<0.05 compared to saline.
3.4 Discussion

The first section of this study investigated the use of HRV in a rat model. As these are conscious free-moving rats, it is important to define the correct parameters for frequency domain analysis. The frequency bands used in conscious rat models vary substantially, as a consequence of age, sex and breed of the rat, as well as time of recording (Table 3.4.1). In this chapter, the low frequency peak was determined as 0.13 Hz and is supported by prior material published by Gomes et al. (2000, 2002). High frequency peaks were measured at 1.4 Hz, similar to Kuwahara et al. (1994) and Rubini et al. (1993). The daytime 5 minute SDNN and LF/HF were consistent with previous literature and supports sympathetic dominance in rats (Cerutti et al., 1991; Kuwahara et al., 1994; Aubert et al., 1999; Hashimoto et al., 1999; Imai et al., 2008). A LF/HF ratio 2-3-fold higher than the present study has been previously reported which may be consequent to the selection of a wider LF band (0.04-1 Hz) by the authors (Kuwahara et al., 1994; Hashimoto et al., 1999).

This chapter clearly demonstrates that KA induced seizures results in altered autonomic activity, particularly elevated parasympathetic modulation. Seizure induction produced bradycardia and low seizure behaviours which were associated with an increase in HF activity, shifting the LF/HF ratio in favour of elevated parasympathetic modulation. The concept of vagal dominance was supported by Sakamoto et al. (2008) where KA administration (10-12 mg/kg, ip, ia or iv) in urethane-anesthetised rats produced a sustained increase in vagal nerve activity. Hotta et al. (2009) reported a drop in HR associated with decreased sympathetic nerve activity in unventilated anesthetised KA dosed animals. Conversely, penicillin-induced seizure in anesthetised rats decreased vagal activity (128-1454%), without affecting HR suggesting that a sympathetic counter-effect may have occurred (Mameli et al., 2001). This is supported by Goodman et al, who had earlier reported activation of both the parasympathetic and sympathetic systems in the amygdaloid kindling model (Goodman et al., 1990; Goodman et al., 1999).

In clinical studies altered autonomic activity and HRV have commonly been reported. Sathyaprabha et al. (2006) reported autonomic dysfunction in 88% of epileptic patients, with increased parasympathetic activity most commonly observed (55%). A reduction in HRV (SDNN, HF and LF) has been reported TLE patients, reflecting cardiovascular autonomic dysfunction (Tomson et al., 1998; Ansakorpi et al., 2002). Conversely, generalised tonic-clonic seizures produced a reduction in HF power and elevated LF activity, demonstrating sympathetic dominance (Evrengul et al., 2005).
Table 3.4.1. Summary of reports on frequency domain analysis in rats

<table>
<thead>
<tr>
<th>Breed</th>
<th>LF band (peak)</th>
<th>HF band (peak)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japundzic et al., 1990</td>
<td>Wistar (310-350g)</td>
<td>0.02-0.2</td>
</tr>
<tr>
<td>Stauss et al., 1995</td>
<td>WKY/SD (10-12 weeks)</td>
<td>0.02-0.2</td>
</tr>
<tr>
<td>Han et al., 2014</td>
<td>Cypl1a1-Ren2 (250-400g)</td>
<td>0.04-0.4</td>
</tr>
<tr>
<td>Murphy et al., 1991</td>
<td>WKY (4-6 months)</td>
<td>0.06-0.2</td>
</tr>
<tr>
<td>Yang et al., 2003</td>
<td>WKY (200-250g)</td>
<td>0.06-0.6</td>
</tr>
<tr>
<td>Kuo et al., 2005</td>
<td>SD (300-430g)</td>
<td>0.06-0.6</td>
</tr>
<tr>
<td>Baltatu et al., 2001</td>
<td>SD (5 months)</td>
<td>0.07-0.3</td>
</tr>
<tr>
<td>Mager et al., 2006</td>
<td>SD (2.5 months)</td>
<td>0.2-0.75</td>
</tr>
<tr>
<td>Cerutti et al., 1991</td>
<td>Lyon rats (14 weeks)</td>
<td>0.27-0.74</td>
</tr>
<tr>
<td>Fazan, Jr. et al., 1999</td>
<td>Wistar (200-350g)</td>
<td>0.015-0.25</td>
</tr>
<tr>
<td>Soares et al., 2004</td>
<td>Wistar (200-300g)</td>
<td>0.2-0.75</td>
</tr>
<tr>
<td>Cerutti et al., 1994</td>
<td>SD</td>
<td>0.27-0.74 (0.34)</td>
</tr>
<tr>
<td>Julien et al., 1995</td>
<td>SD (13 weeks)</td>
<td>0.27-0.74</td>
</tr>
<tr>
<td>Aubert et al., 1999</td>
<td>Wistar (280-300g)</td>
<td>0.19-0.74</td>
</tr>
<tr>
<td>Kruger et al., 1997</td>
<td>SD (230-275g)</td>
<td>0.5-0.8</td>
</tr>
<tr>
<td>Hashimoto et al., 1999</td>
<td>Wistar (250-300g)</td>
<td>0.04-1</td>
</tr>
<tr>
<td>Imai et al., 2008</td>
<td>SD (225-350g)</td>
<td>0.04-1</td>
</tr>
<tr>
<td>Kuwahara et al., 1994</td>
<td>Wistar (12-14 weeks old)</td>
<td>0.04-1 (0.6)</td>
</tr>
<tr>
<td>Rubini et al., 1993</td>
<td>SD (350-400g)</td>
<td>(0.43 Hz)</td>
</tr>
<tr>
<td>Gomes et al., 2002</td>
<td>Wistar</td>
<td>(0.1)</td>
</tr>
<tr>
<td>Gomes et al., 2000</td>
<td>Wistar (210-285g)</td>
<td>(0.1)</td>
</tr>
</tbody>
</table>

Studies using frequency domain analysis in conscious unrestrained rats. The low frequency (LF) band and high frequency (HF) band used for analysis in each study. The brackets represent what frequency the peak (highest power) was observed. SD: Sprague-Dawley, WKY: Wistar Kyoto

Pharmacological modulation of the autonomic system, with ipratropium or atenolol, in this chapter provides insight into the effect of KA-induced HR changes. Ipratropium effectively reduced seizure-induced bradycardia while atenolol reduced the extent of tachycardia observed during seizures. Ipratropium administration also inhibited parasympathetic activity as demonstrated by a drop in the SDNN and HF during the baseline period but did not alter the HRV consequences of seizure activity. Studies looking at the effects of inhaled ipratropium on HRV have reported no change, most likely due to a lack of systemic distribution with this route (Dagnone & Parlow, 1997). Atropine, a hydrophobic analogue of ipratropium, results in a dose-dependent increases in HR (Morrison & Pearson, 1989; Pichot et al., 1999). HRV analysis in free-moving Wistar rats, found that atropine administration significantly increased HR while decreasing power in both the LF and HF bands (Murphy et al., 1991; Aubert et al., 1999). Similar to the present study, Kawahara et al (1994) reported an atropine-induced (2 mg/kg, iv) drop in LF power and HF power by 83% and 78%, respectively, which coincided with an increase in HR and reduced SDNN. Comparable effects are observed in healthy subjects, where atropine administration provokes a drop in RR interval, corresponding to reduced LF and HF power, shifting the LF/HF ratio in favour of the parasympathetic system (Pichot et al., 1999). It should
be noted that because ipratropium is a non-selective muscarinic antagonist, it may alter the LF power via inhibition of M₃ receptor mediated vasodilation resulting in attenuation of the baroreflex modulation of HR (Ren et al., 1993).

Atenolol is a sympatholytic which blocks cardiac β₁ receptors and alters HRV activity. As demonstrated in this study, atenolol significantly increased the RR interval, RR variance and the HF power while decreasing LF power (Sandrone et al., 1994). Atenolol (1 mg/kg, iv) administration decreased HR and LF power in free-moving Wistar rats (Murphy et al., 1991). Previous studies examining the effects of propranolol (4 mg/kg) on HRV in conscious unrestrained rats showed that β-blockade significantly decreased LF power (Kuwahara et al., 1994; Aubert et al., 1999). In normal subjects, atenolol increased the mean RR interval by 24%, due to decreased LF (84%) and increased HF (45%) power (Cook et al., 1991) supporting earlier studies using propranolol (Pagani et al., 1986).

The results from this chapter demonstrate that seizure activity leads to uncontrolled activation of cardiac function with parasympathetic dominance prevalent following systemic KA. Interestingly, this hyperactivation of the parasympathetic system was not completely blocked with ipratropium. It is possible that the dose of ipratropium used in the present study was not high enough, however all rats in the ipratropium group had HRs above 500 b.p.m with a maximum of 602 b.p.m. recorded (74% increase from pre-treatment). Consequently, higher doses of ipratropium were avoided due to the risk of fatal tachyarrhythmias. By reducing the extent of the bradycardia, ipratropium may have attenuated the development of subsequent tachycardic responses during seizure. Close examination of the literature suggests that it is more plausible that ipratropium dosing may have elicited maximum sustainable HR responses (Kikis et al., 1982; Kaya et al., 2004) thereby preventing any further increase in co-ordinated contractile function during seizure. Interestingly, this current study also suggests that in addition to the seizure-induced tachycardia seen, there is also the possibility of a reciprocating consequence of HR on seizure severity, as atenolol significantly reduced seizure progression and high level seizure behaviours while ipratropium produced a significantly higher cumulative behavioural score. The effects of atenolol on seizure activity will be examined further in Chapters 4 and 6 of this thesis.

This is the first study to show the effect of HRV in an animal model of seizure. Atenolol was effective at reducing seizure severity as well as preventing the development of extremely high heart rates and offers a promising cardioprotective strategy in seizure which will be further examined in this thesis. Although ipratropium reduced the extent of HR changes, the tachycardic effect of this drug is likely to increase the severity of seizure pathology.
Chapter 4

Prophylactic treatment with sympatholytics in seizure-induced cardiomyopathy
4.1 Introduction

The previous experimental chapters have clearly demonstrated that seizure activity results in altered cardiac autonomic control, contributing to seizure-induced cardiomyopathy. Cardiac morphology at 48 hours revealed evidence of hypercontracture band necrosis and reversible ischaemic damage, suggesting the development of a sympathetic surge occurring subsequent to the initial parasympathetic mediated bradycardia. This assumption is supported by prior clinical studies where generalised seizure activity primarily results in sympathetic dominance (Evrengul et al., 2005; Mativo et al., 2010).

Current clinical interventions with anti-epileptic agents fail to address the cardiac repercussions of seizure activity. Atenolol is a hydrophilic β₁ antagonist which acts directly on the heart to block the effect of sympathetic stimulation (de Lange et al., 1994; Smith & Teitler, 1999). Clinical studies have also shown that atenolol is effective at attenuating ischaemic damage and improving left ventricular function (Rossi et al., 1983; Mangano et al., 1996; Rousseau et al., 1996; Freemantle et al., 1999; Wallace et al., 1998). Acute administration of atenolol has been shown to prevent tachycardia and protect against seizure-induced QTc prolongation in a rat model of SE (Bealer et al., 2010; Little and Bealer; 2011). Chapter 3 demonstrated that acute atenolol treatment improved heart rate variability and reduced seizure severity. Similarly, the α₂ adrenergic and imidazoline receptor agonist, clonidine has been shown to reduce noradrenaline release whilst increasing vagal activity, thereby simultaneously producing sympatholytic and parasympathomimetic effects (Matsukawa et al., 1995; Azevedo et al., 1999; Toader et al., 2008).

This chapter examines the hypothesis that cardiomyopathy consequent to the development of SE, occurs at least in part, from a centrally-evoked activation of cardiac sympathetic nerves and that seizure-induced cardiac dysfunction and damage may be mitigated by prophylactic treatment with the adrenergic modulators, atenolol or clonidine. The central effect of clonidine will be compared with the cardio-selective properties of atenolol, as we hypothesise clonidine will produce superior therapy through dual neuro- and cardio-protective mechanisms.
4.2 Methods

4.2.1 Materials. All reagents were purchased from BDH (Palmerston North, New Zealand) and Sigma-Aldrich (Auckland, New Zealand). KA (Tocris, Bristol, UK), clonidine (Sapphire Bioscience PTY; New South Wales, Australia) and atenolol (Sigma-Aldrich, NZ), were dissolved in normal saline. Animal prescription remedies were obtained from the University of Otago Animal Welfare Office.

4.2.2 Animals. Sprague-Dawley rats (24 males; 320-350g) were obtained from the University of Otago Animal Resource Unit. The animals were housed on a 12 hour light/dark cycle at 22°C with food and water ad libitum. Experiments were performed in accordance with the University of Otago’s Committee on Ethics in the Care and Use of Laboratory Animals and the “Use of Laboratory Animals” (NIH Publication No. 85-23, 1996).

4.2.3 Experimental Protocol. All animals were implanted with ECG and EEG radiotelemeters (as described in Chapter 2.2.4). Animals were randomised into saline-, atenolol- or clonidine-pre-treated KA groups (n=8 per group). Saline, atenolol (5 mg.kg-1, sc daily; Appendix 8.6; Harris & Aston-Jones, 1993) or clonidine (0.1 mg/kg, twice daily, sc; Zhang & Cheng, 2000; previously used in our laboratory Andreianova, 2011) were administered three days prior to the seizure induction and for the duration of the experiment (n=8/group).

4.2.4 Seizure Induction and Telemetric/Behavioural Recordings. EEG, ECG and behavioural data were simultaneously recorded during a 20 minute baseline period and for 3 hours post-KA administration, with periodic recordings taken at 24 and 48 hours (as described in Chapter 2.2.4). Seizures were induced by a single injection of KA (10 mg/kg, sc, maximum volume 0.35 mL). Following treatment, rats were immediately returned to the chamber for behavioural observation. Behaviour was recorded every 15 seconds for 3 hours with discrete changes in behavioural state additionally reported as they occurred. Behaviours were recorded using a 5-point scale as previously described (Chapter 2.2.5, Table 2.2.1; Hesp et al., 2007; Sawant et al., 2010).

4.2.5 Morphological Characterisation of Myocardial Injury. Hearts were excised 48 hours following KA administration, sectioned and stained with Martius scarlet blue (MSB) (as described in Chapter 2.2.8). Micrographs (10x) of the left ventricular subendocardium (6 mm from apex) were randomly and blindly taken using an Axioplan-2 microscope and semi-quantitative analysis was conducted using Adobe Photoshop CS5 software, in which the ratio of collagen stained (blue) or oedema (white) pixels were calculated against background in each
cardiac section. Each section was examined for evidence of morphological changes, hypercontracture band necrosis and myocyte vacuolisation.

**4.2.6 Data Analysis.** The cumulative score was determined as the sum of the maximum score every minute over the 3 hour recording period. The total number of WDS, Level 4 and Level 5 behaviours were quantified over the 180 minute recording period. Behavioural data was also expressed as the mean behavioural score every 10 minutes. EEG data was analysed, as described in Chapter 2.2.6, using Fast Fourier transformation (FFT) to quantify the frequency bands: delta to beta over 10 minute bins. Baseline PSD for each animal was determined and expressed as a percentage of baseline. High amplitude EEG spiking was defined as sharp electrographic events greater than three-times baseline voltage and quantified using LabChart Spike Analysis over 10 minute bins. Movement artefacts were identified on the ECG trace and eliminated from the EEG analysis. ECG data was analysed using LabChart6 Pro ECG Analysis module software to assess heart rate (HR), QTc intervals and T wave amplitudes (as described in Chapter 2.2.7). Data were analysed every two minutes in one minute blocks over the 3 hour observation period. HRV was analysed every 30 minutes over a 5 minute period using the LabChart6 Pro HRV Analysis module software (as described in Chapter 3.2.4). Statistical analysis was performed using Prism™ v.5 (GraphPad, San Diego, USA). Behavioural data were analysed using a Kruskal-Wallis test with Bonferroni post-hoc. EEG and ECG variables were analysed using a 2-way repeated measures ANOVA with Bonferroni post-hoc analysis. Statistical significance was determined as $P<0.05$. Data are presented as mean ± standard error of the mean (SEM).
4.3 Results

4.3.1 Seizure Activity

In this study, KA administration in the saline pre-treated group resulted in an immediate increase in seizure behaviours with WDS’s developing 47.3 ± 8.1 minutes post-KA and progressing to Level 4 behaviours at 70.0 ± 8.8 minutes (Table 4.3.1). Pre-treatment with atenolol and clonidine had no significant effect on the latency of onset to hypoactivity, Level 2 or WDS behaviours, although clonidine significantly decreased the onset to Level 4 behaviours occurring 47.6 ± 5.4 minutes post-KA (P<0.05 compared to saline).

Table 4.3.1. Latency to seizure behaviour onset

<table>
<thead>
<tr>
<th></th>
<th>Saline (min)</th>
<th>Atenolol (min)</th>
<th>Clonidine (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoactivity/Abnormal resting</td>
<td>6.3 ± 1.2</td>
<td>7.3 ± 0.99</td>
<td>4.4 ± 0.98</td>
</tr>
<tr>
<td>Level 2 behaviours</td>
<td>11.6 ± 4.9</td>
<td>20.8 ± 6.8</td>
<td>6.2 ± 1.3</td>
</tr>
<tr>
<td>WDS</td>
<td>47.3 ± 8.1</td>
<td>39.3 ± 6.0</td>
<td>39.9 ± 5.8</td>
</tr>
<tr>
<td>Level 4</td>
<td>70.0 ± 8.8</td>
<td>64.9 ± 5.7</td>
<td>47.6 ± 5.4 *</td>
</tr>
</tbody>
</table>

Time taken to first seizure behaviour following KA (10 mg/kg). * P<0.05 compared to Saline

KA administration in saline pre-treated animals, resulted in a progressive increase in behavioural score which correlated with an increase in high amplitude EEG spiking and power (theta-beta frequency bands, Figure 4.3.1 and 2). Atenolol pre-treatment reduced the development of seizure progression 120-180 minutes post-KA (Figure 4.3.2C) and decreased the cumulative score by 31.6% compared to the saline group (P<0.05, Figure 4.3.1). Atenolol pre-treatment successfully attenuated the effect of KA on EEG power in the delta to beta frequency bands (P<0.05 compared to Saline, Figure 4.3.2A), as well as decreasing high amplitude spiking (Figure 4.3.2B). Pre-treatment with clonidine reduced seizure severity 150-180 minutes post-KA and reduced the development of WDS by 64% (P<0.05 compared to saline; Figure 4.3.1 and 2C). Clonidine administration did not produce any significant changes in the EEG power during seizure, but decreased high amplitude spiking by ~90% at 160-180 minutes post-KA administration, compared to the time matched saline group (P<0.05, Figure 4.3.2B). Level 5 behaviours occurred in 5 out of 7 saline rats, while only 2 in the atenolol group and 1 in the clonidine group developed Level 5 behaviours. There was no significant increase in seizure behaviours or EEG activity at the 24 and 48 hour recording periods in any group.
**Figure 4.3.1.** Behavioural changes during KA induced seizure activity over 180 min. Cumulative score (A), number of wet dog shake (WDS, B) and Level 4 behaviours (C) following KA (10 mg/kg). Values represent mean ± SEM. *P<0.05 compared to Saline-KA.

**Figure 4.3.2.** EEG and behavioural changes following seizure induction over 180 min (mean ± SEM). (A) Power spectral density (PSD): Histograms represent normalised PSD for each 10 min bin across the delta (1.25-4.5 Hz), theta (4.75-6.75 Hz), alpha (7.0-12.5 Hz) and beta (12.75-35.0 Hz) frequency bands. (B) Number of high amplitude EEG spikes in a 10 min bin. (C) Mean behavioural score every 10 min. *P<0.05 compared to Baseline. *P<0.05 compared to saline treatment.
4.3.2 Cardiac function

KA administration in saline pre-treated animals, produced an immediate period of bradycardia with tachycardia developing 60-124 minutes post-KA. These animals also had significant increases in QTc interval and T wave elevation during the 180 minutes post-KA. HRV analysis at 24 and 48 hours revealed an increase in the LF/HF ratio by 34% and 25%, respectively (Figure 4.3.6). Consistent evidence of ventricular oedema, myocyte vacuolisation, inflammatory cell infiltration and fibrosis were seen in the saline pre-treated hearts 48 hours seizure-induction (Figure 4.3.8; Table 4.3.2).

Pre-treatment with atenolol decreased HR to 308 ± 10 b.p.m. (10%) during the baseline period (Figure 4.3.3). Atenolol administration reduced the extent of HR changes (HR ranged from 233-362 b.p.m during seizures) and completely prevented the development of tachycardia seen in the saline group (P<0.05 at 50-140 minutes). Atenolol successfully attenuated QTc prolongation during seizures, with a maximum increase of 17% recorded (compared to 39% in the saline group, P<0.05, Figure 4.3.4). Pre-treatment with atenolol completely prevented elevation of the T wave (132-180 minute; P<0.05, Figure 4.3.5). Atenolol pre-treatment significantly reduced the incidence of arrhythmias, which were observed during the initial 30 minutes post-KA in the saline animals (14% had PVC and 42% have AV block compared to saline treated animals, as described in Chapter 2.2.2.). HRV analysis revealed an increase in SDNN (by 53-82%) and TP (559-645%), 15-30 minutes post-KA in atenolol pre-treated animals compared to baseline (Figure 3.3.6). Atenolol pre-treatment prevented tachycardia which was associated with an increase in HF activity 150-180 minutes post-KA. Atenolol pre-treatment also prevented the sympathetic dominance observed 24 and 48 hours post-KA in the saline treated (Figure 4.3.6C). Atenolol treatment preserved normal cardiac morphology 48 hours post-KA (Figure 4.3.7). There was a significant decrease in fibrosis, reduced occurrence of hypercontracture band necrosis and reversible ischaemic damage (Table 4.3.2).

Clonidine pre-treatment reduced HR from 338 ± 11 b.p.m to 267 ± 13 b.p.m. (P<0.05, Figure 4.3.3). Administration of KA resulted in a further decrease in HR to 197 ± 12 b.p.m. at 18 minutes. Clonidine pre-treatment significantly reduced the tachycardia recorded in the saline group during high level seizure behaviours (50-140 minutes post-KA, P<0.05 Figure 4.3.2C and 4.3.3). Disappointingly, clonidine did not offer any significant protection against bradyarrhythmias (all 8 animals exhibited evidence of PVC and 4 had AV block).
Figure 4.3.3. Heart rate (beats per minute, b.p.m.) changes following saline, atenolol or clonidine prior to seizure induction with KA (dashed line). Mean ± SEM. *P<0.05 compared to mean baseline. *P<0.05 compared to saline treatment.

Figure 4.3.4. Corrected QT interval (seconds) following saline, atenolol or clonidine prior to seizure induction with KA (dashed line). Mean ± SEM. *P<0.05 compared to mean baseline, *P<0.05 compared to saline treatment.
Clonidine significantly prevented QTc prolongation and reduced the extent of T wave elevation 148 to 180 minutes post-KA ($P<0.05$ compared to saline), with maximum increases of 30-40% above baseline. Clonidine pre-treatment significantly altered the balance in autonomic function, prior to KA administration (Figure 4.3.6/7). There was a significant increase in TP by 8-fold associated with a drop in LF to $32 \pm 6$ nu compared to $67 \pm 4$ nu in the pre-treatment recording. KA administration resulted in a significant increase in SDNN and total power by 125% and 215%, respectively, at 15 minutes. Clonidine pre-treatment was also effective at preventing sympathetic dominance at 24 and 48 hours (Figure 4.3.7C). Pre-treatment with clonidine reduced the extent of fibrosis, hypercontracture band necrosis and myocyte vacuolisation, but did not prevent oedema (Table 4.3.2).

![Figure 4.3.5. T wave amplitude changes following saline, atenolol or clonidine prior to seizure induction with KA (dashed line). T wave normalised to mean baseline. Mean ± SEM. #P<0.05 compared to mean baseline, *P<0.05 compared to saline treatment.](image)
Table 4.3.2. Relative extent of morphological features at 48 hours post-seizure onset in left ventricular subendocardial regions.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Saline</th>
<th>Atenolol</th>
<th>Clonidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrosis</td>
<td>0.9 ± 0.3 %</td>
<td>0.16 ± 0.02 *</td>
<td>0.14 ± 0.06 *</td>
</tr>
<tr>
<td>Oedema</td>
<td>31 ± 5.0 %</td>
<td>19 ± 3.9</td>
<td>24 ± 3.8</td>
</tr>
<tr>
<td>Myocyte vacuolisation</td>
<td>7.7 ± 1.1</td>
<td>1.5 ± 0.7 *+</td>
<td>4.3 ± 0.3*</td>
</tr>
<tr>
<td>Hypercontracture band necrosis</td>
<td>6.2 ± 1.1</td>
<td>0.5 ± 0.3 *</td>
<td>2.6 ± 0.4 *</td>
</tr>
</tbody>
</table>

Quantification of each feature was conducted on 10 fields from ventricular sections. Fibrosis and oedema were quantified as the number of positively stained pixels per field (%). Myocyte vacuolisation and hypercontracture band necrosis were graded on the presence of 2+ vacuolisation nuclei positive cells in each field. *P<0.05 compared to Saline, +P<0.05 compared to clonidine.

Figure 4.3.6. Comparison of the effect of atenolol and clonidine on heart rate variability in seizure. Standard deviation of normal RR intervals (SDNN, milliseconds; A), total power (ms², B), low frequency (LF; 0.04-0.5 Hz, C), high frequency (HF; 0.5-3 Hz, D) and LF/HF ratio (E) following treatment with saline, atenolol or clonidine prior to KA administration. Data represents in normalised units, mean ± SEM. *P<0.05 compared to baseline. *P<0.05 compared to saline treatment.
Figure 4.3.7. Representative micrographs of left ventricular subendocardium collected at 48 h following (A) saline administration to control animals showing normal representative myocardium; (B-F) Effects of KA administration showing: (B) myocyte vacuolisation (asterisks indicate intracellular vacuoles displacing nuclei) and oedema; (C) Coagulative myocytolysis illustrated by hypercontraction band necrosis associated with fibre derangement (arrowheads) and fibrosis; (D) Inflammatory necrosis encapsulated by collagen deposition (blue fibres) indicative of early restorative fibrosis. Hearts from atenolol (E) and clonidine (F) pre-treated animals showing preserved normal cardiac morphology.
Figure 4.3.8. Time course comparing the effect of atenolol and clonidine on ECG, EEG and behavioural changes during seizure induction in individual animals. Representative traces showing combined heart rate (b.p.m.; black line trace), EEG activity (mV; grey spectral trace) and behavioural score responses following KA (grey line) administration in individual rats pre-treated with saline, atenolol or clonidine.
4.4 Discussion

This study clearly demonstrates that seizure activity provokes cardiac dysfunction and injury, most likely as a consequence of altered autonomic activity. Pre-treatment with atenolol was effective at reducing seizure severity (EEG and behaviour) and ECG changes, as well as preserving cardiac morphology. Clonidine significantly reduced high level seizure behaviours and protected against tachycardia, QTc prolongation and T wave elevation.

In this study, pre-treatment with atenolol was effective at reducing seizure severity and progression, as well as EEG activity following KA administration. This was unexpected as atenolol is a hydrophilic β1 receptor blocker reported to have a very low permeability (brain:plasma ratio of 0.2) through the blood brain barrier (Patel & Turner, 1981; Drayer, 1987). Older lipophilic β blockers, such as metoprolol and propranolol (Table 4.1), have been previously shown to produce anticonvulsant effects in multiple seizure animal models while atenolol offered no seizure protection. The non-selective β1 and β2 antagonist, propranolol (1-20 mg/kg), has been shown to increase the threshold to seizure induction in a variety of seizure models (Fischer et al., 1985; De Sarro et al., 2002a; Fischer, 2002; Nakamura et al., 2008). Metoprolol and nebivolol, selective β1 antagonists, decrease the development of tonic-clonic seizures and increase the threshold and latency to seizure onset in experimental seizure. In a previous study by Little and Bealer (2011), a single bolus injection of atenolol (1 mg/kg, iv) had no influence on EEG activity or seizure behaviours in rats with electrically induced SE. The same dose of atenolol offered no protection against seizure-induced hippocampal damage in pilocarpine-induced SE (Bealer et al., 2010). Interestingly, De Sarro et al. (2002) gave high doses of acute atenolol (up to 50 mg/kg) and still reported no anti-convulsant effects. It is possible that the chronic administration of a high dose of atenolol used in the present study allowed a small percentage of the atenolol dose to cross into the brain where it could inhibit proconvulsant neuronal β1 receptors in the hippocampus (Mueller & Dunwiddie, 1983). An alternative explanation is that by preventing the development of tachycardia and maintaining cardiac output, atenolol reduced the extent of cerebral hypoxia decreasing consequent cerebral hyperexcitabilty (Morady et al., 1985; Kobari et al., 1992; Ocon et al., 2009). Previous studies using atenolol, may have found no anticonvulsant effect due to the different experiment design. De Sarro et al. (2002) quantified the percentage of rats presenting with seizures at 60 minutes. Comparatively in the current study, all rats presented with seizures, however the frequency was reduced in atenolol treated animals. The lack of protection observed in Little and Bealer’s (2011) study may be a consequence of only a brief analysis window (5 minutes), compounded by a short experimental time frame (up to 90 minutes). In this thesis
Atenolol produced decreased seizure severity 150-180 minutes post seizure induction which is outside the time frame of previous studies examining beta blockade.

**Table 4.4.1.** Summary of reported effects of beta blockers on seizure generation in animal studies

<table>
<thead>
<tr>
<th>Drug (dose)</th>
<th>Epilepsy Model</th>
<th>Effect on seizure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anticonvulsant effect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lathers <em>et al.</em>, 1989</td>
<td>Timolol 1-20 mg/kg, iv</td>
<td>Cats: PTZ Suppressed epileptic activity</td>
</tr>
<tr>
<td>De Sarro <em>et al.</em>, 2002a</td>
<td>Propranolol, metoprolol, 30-50 mg/kg, ip</td>
<td>Mice: Autogenic Propranolol: ↓ clonic and tonic seizures Metoprolol: ↓ tonic, clonic and wild running phase of seizures</td>
</tr>
<tr>
<td>Fischer, 2002</td>
<td>Propranolol 2-20 mg/kg, ip</td>
<td>Mice: Variety Dose-dependent ↑ in threshold of electroshock seizures. Suppressed tonic-clonic convulsions in chemical seizures</td>
</tr>
<tr>
<td>Nakamura <em>et al.</em>, 2008</td>
<td>Propranolol 1-3 mg/kg, iv</td>
<td>Rats: Lidnocaine ↑ in threshold to seizures</td>
</tr>
<tr>
<td>Goel &amp; Goel, 2013a</td>
<td>Carvediol 1.25-5 mg/kg, po</td>
<td>Mice: Electroshock or PTZ ↑ seizure threshold current at all doses. ↑ latency to seizure following PTZ</td>
</tr>
<tr>
<td>Goel &amp; Goel, 2013b</td>
<td>Nebivolol 0.5 mg/kg</td>
<td>Mice: PTZ ↑ seizure threshold and latency</td>
</tr>
<tr>
<td><strong>No neuronal effect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>De Sarro <em>et al.</em>, 2002a</td>
<td>Atenolol 1-50 mg/kg, ip</td>
<td>Mice: Autogenic Atenolol had no protective effect on seizure activity</td>
</tr>
<tr>
<td>Bealer <em>et al.</em>, 2010</td>
<td>Atenolol 1 mg/kg, iv</td>
<td>Rats: Pilocarpine Did not protect against hippocampal damage</td>
</tr>
<tr>
<td>Little &amp; Bealer, 2012</td>
<td>Atenolol 1 mg/kg, iv</td>
<td>Rats: Electroshock No effect on seizure severity</td>
</tr>
</tbody>
</table>

*Anticonvulsant effect of beta blockers in animal models of seizure. Propranolol and timolol are non-selective β antagonists. Atenolol, metoprolol and nebivolol are β1 selective antagonists. Carvediol is an antagonist of β1, β2 and α1 receptors (Kendall, 1997). PTZ= pentylenetetrazol*

Atenolol is a rate-control anti-arrhythmic β1 antagonist which decreases cardiac sympathetic stimulation (de Lange *et al.*, 1994; Smith & Teitler, 1999). Atenolol successfully reduced the development of bradyarrhythmias, tachycardia, QTc prolongation and T wave elevation in association with the preservation of cardiac morphology. There was very little evidence of myocyte vacuolisation and no hypercontraction band necrosis observed in the atenolol treated hearts. Atenolol also prevented the development of fibrosis and reduced the extent of oedema in these hearts. Clinical studies have revealed that atenolol is effective at reducing mortality and morbidity during sympathetic surge in post-myocardial infarction and non-cardiac surgery (Rossi *et al.*, 1983; Mangano *et al.*, 1996; Wallace *et al.*, 1998; Freemantle *et al.*, 1999). Atenolol (100 and 200 mg, po) is rapidly absorbed with reductions in HR and systolic pressure observed within 30 minutes and persisting for up to 8 hours (Fitzgerald *et al.*, 1978). In rat models of SE, atenolol (1 mg/kg, iv) preserved cardiac output, protected against QTc prolongation and reduced cardiac...
damage (Bealer et al., 2010; Little and Bealer, 2011). Atenolol has also been used following head trauma to prevent an elevation in cardiac enzymes, ECG abnormalities and arrhythmias (Cruickshank et al., 1987; Cruickshank et al., 1988). These results demonstrate that atenolol has the potential to offer enhanced protection in epileptic patients and should be considered as a combination therapy with other antiepileptic agents.

In the present study, clonidine pre-treatment altered the seizure activity induced by KA and effectively reduced high amplitude spiking and WDS behaviours, however it did not reduce the power of the EEG frequencies. Clonidine previously been reported to decrease WDS, protect against limbic seizures and prevent neurochemical changes (Table 4.2; Kleinrok & Turski, 1980; Baran et al., 1985; Ohno et al., 1987; Yoshioka et al., 2000b; Baran et al., 2000; Read et al., 2014). The findings in the present study are in agreement with previous research suggesting that clonidine has anticonvulsant effects which appear to be mediated by the α2 adrenoreceptor. Alpha-2 receptor agonists such as dexmedetomidine, effectively reduce the development, generalisation and severity of KA-induced seizures while selective α2 antagonists such as yohimbine and atipamezole, have a pro-convulsant effect and are associated with increased mortality (Ohno et al., 1987; Halonen et al., 1995).

Conversely, studies have also shown that clonidine may have pro-convulsant effects which appear to be age- and dose-dependent (Yoshioka et al., 2000a; Szot et al., 2004; Feron et al., 2008). Szot et al. (2004) hypothesised that α2A pre-synaptic autoreceptors mediate the pro-convulsant effects of clonidine while α2A post-synaptic receptors are responsible for the anticonvulsant effects. Clonidine has a 10 fold higher affinity for pre-synaptic α2 adrenoreceptors than it does for the post-synaptic receptors (Maura et al., 1985), which suggests that a high dose of clonidine is required to achieve the anticonvulsant effects. This disputed earlier studies by Papanicolaou et al. (1982) and Jackson et al. (1991) who reported a U shaped dose trend with doses ≤ 0.1 mg/kg producing anti-convulsant effects, while larger doses resulted in a dose-dependent decrease in the anticonvulsant effects of clonidine. The age of the animal also appears to influence the effect of clonidine with young animals more susceptible to the pro-convulsant effects of clonidine (Yoshioka et al., 2000a). Table 4.2 demonstrates the complexity of convulsant responses to clonidine which may be dependent on subject age and the seizure model as well as dosing regimen. Chronic dosing with 0.1 mg/kg of clonidine in the current study, produced a significant reduction in seizure severity.
### Table 4.4.2. Summary of reported effects of clonidine on seizure induction in rodent models.

<table>
<thead>
<tr>
<th>Dose (mg/kg, ip)</th>
<th>Model</th>
<th>Effect on seizure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-convulsant effect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papanicolaou et al., 1982</td>
<td>0.0001-0.1 Rats: PTZ</td>
<td>Dose-dependent ↓ in duration of seizures</td>
</tr>
<tr>
<td>Jackson et al., 1991</td>
<td>0.05 Mice: electroshock</td>
<td>↑ threshold</td>
</tr>
<tr>
<td>Baran et al., 1985</td>
<td>0.1 Rats: KA</td>
<td>↓WDS and if given for 3 days prior it almost completely abolished WDS</td>
</tr>
<tr>
<td>Velisek et al., 1994</td>
<td>0.25 Rats: KA</td>
<td>↓WDS and tonic-clonic seizures ↑ latency to tonic-clonic seizures</td>
</tr>
<tr>
<td>Amabeoku, 1993</td>
<td>0.25-1 Mice: Imipramine</td>
<td>Dose-dependently delayed the onset of tonic seizures.</td>
</tr>
<tr>
<td>Lazarova et al., 1984</td>
<td>0.5 Rats: PTZ</td>
<td>↓ tonic seizures and mortality, ↑ latency</td>
</tr>
<tr>
<td>Tacke &amp; Kolonen, 1984</td>
<td>0.5-1 Autogenic rats</td>
<td>↑ latency to seizure onset</td>
</tr>
<tr>
<td>Wu et al., 1987</td>
<td>0.5-2 Rats: Quinolinic acid</td>
<td>↓ number and total time of seizures</td>
</tr>
<tr>
<td>Amabeoku et al., 1994</td>
<td>1 Mice: PTZ</td>
<td>Delayed onset but no effect on incidence</td>
</tr>
<tr>
<td>Shafaroodi et al., 2013</td>
<td>1-5 Mice: PTZ</td>
<td>↑ threshold</td>
</tr>
<tr>
<td><strong>Pro-convulsant effects</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sitnikova &amp; van, 2005</td>
<td>0.006 Rats: Genetic</td>
<td>↑ total time of seizures</td>
</tr>
<tr>
<td>Wu et al., 1987</td>
<td>0.1 Rats: Quinolinic acid</td>
<td>↑ number of seizures and total time spent in seizures</td>
</tr>
<tr>
<td>Szot et al., 2004</td>
<td>0.1 Mice: Flurothyl</td>
<td>Decreased latency to clonic-tonic seizures</td>
</tr>
<tr>
<td>Fletcher &amp; Forster, 1988</td>
<td>0.63-3.1 Rats: PTZ</td>
<td>↓ threshold</td>
</tr>
<tr>
<td>Fletcher &amp; Forster, 1988</td>
<td>1-10 Mice: PTZ</td>
<td>↓ threshold (no effect 0.08-1 mg/kg)</td>
</tr>
<tr>
<td><strong>No effect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yokoyama et al., 1993</td>
<td>0.0001-0.001 Rats: Lignocaine</td>
<td>No effect on cumulative convulsant doses</td>
</tr>
<tr>
<td>McIntyre &amp; Giugno, 1988</td>
<td>0.0001-0.001 Rats: Lignocaine</td>
<td>No effect on threshold</td>
</tr>
<tr>
<td>Fletcher &amp; Forster, 1988</td>
<td>0.0002-0.13 Rats: PTZ</td>
<td>No effect on threshold</td>
</tr>
<tr>
<td>Amabeoku et al., 1994</td>
<td>0.0125-0.25 Mice: PTZ</td>
<td>No effect on incidence or onset</td>
</tr>
<tr>
<td>Wu et al., 1987</td>
<td>0.05-0.25 Rats: Quinolinic acid</td>
<td>No effect on number of seizures</td>
</tr>
<tr>
<td>Fletcher &amp; Forster, 1988</td>
<td>0.08-1 Mice: PTZ</td>
<td>No effect on threshold</td>
</tr>
<tr>
<td>Jackson et al., 1991</td>
<td>0.1-0.8 Mice: electroshock</td>
<td>No effect on threshold</td>
</tr>
</tbody>
</table>

*KA: kainic acid, PTZ: pentylenetetrazol,*

In addition to reduced seizure behaviours, pre-treatment with clonidine produced significant cardiac protection as observed by prevention of tachycardia, QTc prolongation and T wave elevation. The binding of clonidine at pre-synaptic α2 adrenoreceptors reduces the release of noradrenaline resulting in an attenuation of the cardiac sympathetic response (Matsukawa et al., 1995; Blackman et al., 1996; Ally, 1998; Kofoed et al., 1999; Zhang & Cheng, 2000; Daviss et al., 2008; Champeroux et al., 2010). Pre-treatment with clonidine effectively reduced seizure-induced tachycardia, thereby preserving cardiac morphology and protecting the heart from the development of micro-lesions. Studies specifically examining the risk of QT prolongation in humans and animals have shown that clonidine administration results in an increased RR interval.
Chapter 4: Prophylactic treatment with sympatholytics in seizure-induced cardiomyopathy

but does not alter the baseline QTc interval (Kofoed et al., 1999; Yeragani et al., 2003; Testai et al., 2007; Champeroux et al., 2010). Similarly clonidine has also been reported to attenuate QTc prolongation induced by the arrhythmogenic agents, thioridazine and terfenadine, in dogs (Champeroux et al., 2010). Clonidine acts to decrease HR, BP and noradrenaline levels and decreases mortality and morbidity following heart failure or myocardial infarction in animal and clinical studies (Ally, 1998a; Azevedo et al., 1999; Zhang & Cheng, 2000; Daviss et al., 2008). Clonidine was commonly used for the treatment of hypertension, however it has been largely superseded by the introduction of diuretics, calcium channel blockers and angiotensin modulators. Clonidine reduces sympathetic activity with beneficial effects in peri-operative tachycardia and myocardial ischaemia and can improve survival in patients with chronic heart failure (Ellis et al., 1994; Azevedo et al., 1999; Zhang & Cheng, 2000; Wijeysundera et al., 2009). These joint neuro- and cardio-protective effects of clonidine suggest that it could be used as a prophylactic against cardiomyopathy in seizure. It must be noted however, that the clonidine dosed animals appeared sedated and less responsive, mirroring clinical data commonly reporting sedative effects and dry mouth lasting up to 12 hours following high dose clonidine (Keranen et al., 1978; Hall et al., 2001). These effects may result in reduced compliance to prophylactic courses of clonidine in epileptic patients.

This study clearly demonstrates that sustained seizure activity results in altered cardiac function leading to ventricular myocardial micro-lesions. Prophylactic treatment with atenolol or clonidine was effective at protecting against seizure-induced cardiomyopathy. Both therapies reduced seizure progression and prevented cardiac dysfunction. Atenolol appeared to be well tolerated in this study and was more effective than clonidine at reducing the extent of ischaemic damage seen in these hearts at 48 hours. Prophylactic treatment with clonidine in ischaemic patients may require a more exclusive criteria, with age, dose and compliance strictly monitored. The results obtained in this study clearly show the importance of therapeutically protecting the heart against sympathetic overdrive during the early stages of status epilepticus.
Chapter 5

Cardiac dysfunction following intrahippocampal kainic acid-induced seizures
5.1 Introduction

In the previous chapters, systemic KA administration was shown to produce a reliable reproducible model of limbic seizure behaviours, developing to status epilepticus. Animals display an escalating level of behavioural responses which include hypoactivity, tremors, wet dog shakes and eventually clonic tonic convulsions, persisting for 120-180 minutes (Chapter 2-5; Lothman & Collins, 1981; Chen et al., 2002; Hesp et al., 2007; Sawant et al., 2010; Read et al., 2014). The presence of subunit binding sites for ionotropic glutamate receptors have been found in peripheral tissues such as the heart, kidney, liver, lungs and adrenal glands (Winter & Baker, 1995; Gill et al., 1998; Mueller et al., 2003). Using antibody binding studies, Gill and co-workers were able to show the presence of GluR2/3, KA2 and NMDAR1 subunits associated with ganglia cells, conducting fibres, nerve bundles and cardiomyocytes in the hearts of rats (Gill et al., 1998). At present, however, functional evidence of cardiac glutamate receptor activity remains unavailable. Previous work in our lab, found that direct administration of domoic acid (1-5 μM) or KA (50 μM) to an isolated heart preparation failed to alter HR or left ventricular function (Vranyac-Tramoundanas, 2007). Although these previous results, support the concept that KA has minimal direct cardiac effects, it is still possible that systemic KA may alter cardiac function though indirect mechanisms, such as baroreflex or renin-angiotensin mediated pathways.

In order to selectively study the cardiac consequences of seizure, this chapter investigates seizure-induced cardiomyopathy in a model involving direct administration of KA into the right hippocampus. Prior work confirmed that intrahippocampal delivery of the excitotoxin, domoic acid (200 pmol, 1 μl) did not produce any detectable levels in the plasma (Vranyac-Tramoundanas et al., 2011). This improved model therefore ensures that the cardiomyopathy produced is a consequence of seizure activity. Initially a pilot study was performed to determine the dose of KA required to elicit sustained seizure activity. This investigation revealed that intrahippocampal administration of KA (0.5-2 nmol) produced a dose-dependent increase in seizure scores within the first 3 hours (Appendix 8.1.1). At 48 hours, there was a dose-dependent increase in the extent of myocardial damage and fibrosis. A high dose of KA (2 nmol) produced sustained Level 3 to 4 behaviours which coincided with tachycardia, QTc prolongation and T wave elevation. Interestingly, no bradycardia or behavioural hypoactivity was observed, in this intrahippocampal KA model.

This chapter examines the use of intrahippocampal KA administration on seizure behaviours and cardiac function in a longitudinal study up to 28 days. Additionally, this chapter will further
assess the involvement of the sympathetic system in seizure-induced cardiomyopathy, via plasma noradrenaline, HRV analysis and pharmacological blockade. The consequence of seizure on left ventricular function and structure was evaluated using echocardiography. Previous chapters have demonstrated seizure-induced cardiomyopathy results in micro-lesions, particularly fibrotic deposition, therefore the arrhythmogenic agent, aconitine, was used to assess whether this altered susceptibility to arrhythmia.
5.2 Methods

5.2.1 Materials. All reagents were purchased from BDH (Palmerston North, New Zealand) and Sigma-Aldrich (Auckland, New Zealand). Prescription remedies were obtained from the University of Otago’s Drug Control Officer at the University of Otago Animal Welfare Office. KA was purchased from Tocris (Bristol, UK) and dissolved in saline (0.9% NaCl).

5.2.2 Animals. Sprague-Dawley rats (61 males; 320-350g) were obtained from the University of Otago Animal Resource Unit. The animals were housed on a 12 hour light/dark cycle at 22°C with food and water ad libitum and left to acclimatise for 5 days prior to surgery. Experiments were performed in accordance with the regulations of the University of Otago’s Committee on Ethics in the Care and Use of Laboratory Animals and the “Use of Laboratory Animals (NIH Publication No. 85-23, 1996)”.

5.2.3 Experimental Protocol. All animals had a 26G intra-hippocampal drug cannula (Coherent Scientific, Australia; Figure 5.2.1B) secured into the right hippocampus (5.2 mm posterior of Bregma and 5 mm right of the midline at a depth of 5.2 mm; Figure 5.2.1). A subset of animals, (25 rats) were also implanted with EEG/ECG transmitters (as detailed in Chapter 2.2.4). Following surgery, animals were randomised into treatment groups as shown in Figure 5.2.2. In the pilot study, cannula placement was confirmed in 20 rats (Appendix 8.1.2).

5.2.4 Seizure induction. Following establishment of seizure responses to a range of KA intrahippocampal doses (see Appendix 8.1.1), seizures were induced by an intrahippocampal infusion of KA (2 nmol, 1 μl given over 1 minute), using a Beehive syringe driver (Bioanalytical Systems, West Lafayette, Indiana, USA) and glass Hamilton syringe. All animals were monitored behaviourally for 180 minutes to ensure successful seizure-induction of Level 4 behaviours. All rats were administered saline at 60 minutes to examine the effect of subcutaneous saline on EEG and ECG activity (these seizure rats will be used in Chapter 6). EEG/ECG activity
was recorded in the transmitter rats with behavioural activity recorded every 15 sec (as described in Chapter 2.2.5). EEG data was analysed, as described in Chapter 2.2.6, using Fast Fourier transformation (FFT) to quantify the major frequency bands and normalised to baseline. The cumulative score was determined as the sum of the maximum score every minute over the 180 minute recording period, with the total number of WDS, Level 4 and Level 5 behaviours also noted. ECG data was blindly analysed using LabChart6 Pro ECG Analysis module software to assess heart rate (HR), QTc intervals (as described in Chapter 2.2.7) and T wave amplitudes. Data were analysed every two minutes in one minute blocks over the 180 minute observation period. HRV was analysed over a 5 minute period throughout the study, as described in Chapter 3.2.4. In a subset of animals (n=6/group), arterial systolic BPs were recorded prior to seizure induction and periodically post-KA. Four repeat blood pressure recordings were taken using LabChart and the mean of the systolic blood pressure values determined. In another subset of animals, tail vein blood samples (0.5 mL) were taken over the course of the study to determine noradrenaline and cortisol levels.

**Figure 5.2.2.** Delegation of rats into respective treatment groups, experimental studies and time courses. Ipra/Aten: ipratropium and atenolol (vagal sympathetic effect) study.
5.2.4. **Vagal-sympathetic effect.** Vagal-sympathetic effect (VSE) was calculated from the ratio of intrinsic HR (iHR) to baseline HR (Machado & Brody, 1989; Bealer, 2002; Metcalf *et al.*., 2009b). The use of iHR is defined as HR in the absence of neural influences and represents the basal activity of the cardiac pacemaker. iHR was determined by administration of ipratropium (5 mg/kg, sc) and atenolol (5 mg/kg, sc) to block muscarinic and β1 mediated autonomic control, respectively. Pre-seizure measurements were taken 2-3 days prior to seizure induction. A 30 minute baseline HR was taken followed by either atenolol or ipratropium administration. The HR was recorded for a further 30 minutes, at which time the remaining antagonist was given. Animals were randomly assigned so that half received ipratropium first and half received atenolol first. The experiment was repeated at 48 hours or 7 days following seizure induction (n=6/time point). Reported baseline HR represents the mean HR over the 30 minute recording period prior to drug treatment. The iHR was taken as the mean HR over 30 minutes post combined antagonist treatment. VSE (iHR/baseline HR) values greater than 1, suggest an increase in parasympathetic dominance. Conversely, VSE values less than 1 are indicative of an elevated sympathetic control.

![Diagram showing the protocol used to determine cardiac sympathetic and para-sympathetic tone. Vagal sympathetic effect determined as the ratio of baseline and intrinsic heart rate. Adapted from Damasceno et al., 2013.](image)

5.2.5. **Noradrenaline and cortisol plasma levels.** Tail vein blood samples were taken throughout the experiment and centrifuged for 3 minutes at 3000 r.p.m. The plasma was extracted and frozen (-80°C) until analysis. Plasma noradrenaline levels were determined using a rat noradrenaline enzyme-linked immunosorbent assay (ELISA; Labor Diagnostika Nord, Germany, BA E-5200). Samples (100 μl) were added in duplicate to the antibody coated 96 well plate and left to incubate for 60 minutes at room temperature. Wells were washed (3×) and acylation buffer (150 μl) and acylation reagent (25 μl) added to each well to incubate for 20 minutes. The wells were washed and hydrochloric acid (100 μl for 10 minutes) added to all wells to quench the reaction. The supernatant (90 μl) from each well was removed and added into a new microtitre plate with 25
μl of enzyme solution containing catechol-O-methyl transferase and incubated for 2 hours at 37°C. Following incubation, 100 μl of samples were added to a pre-coated noradrenaline microtitre plate with noradrenaline anti-serum (50 μl) and left for 15 hours at 4°C. The plate was washed and 100 μl of enzyme conjugate was added to all wells and left for 30 minutes at room temperature. The chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB, 100 μl) was added to all wells for 20 minutes at room temperature. The reaction was stopped using stop solution (containing sulphuric acid) and absorbance immediately read at λ = 450 nm (SpectraMax, Molecular Devices, USA).

Plasma cortisol levels were determined using a rat cortisol ELISA (MyBioSource, USA, MBS701698). Sample assays were performed in duplicate using 50 μl of sample added to each antibody coated well and left to incubate for 40 minutes at 37°C. Following washing, 100 μl of horseradish peroxidase-conjugate was added to each well and left to incubate for 30 minutes at 37°C. Following this, TMB (90 μl) was added to each well and incubated for a further 20 minutes in the absence of light. Reaction was terminated using the proprietary stop solution provided and optical density immediately measured (λ = 450 nm). The concentration of cortisol in each well was determined using a standard curve of known cortisol concentrations.

5.2.6. Echocardiography. Rats (n=5/group, Figure 5.5.1) were sedated with domitor and the chest shaved. Left ventricular dimensional and functional parameters were measured across the parasternal long axis view using the Vivid E9 ultrasound system (GE Healthcare). Left ventricular end-systolic and end-diastolic diameters were measured at the level of the papillary muscles using two-dimensional guided M-mode imaging. Measurements of left ventricular dimensions were performed on-line from the recording (Figure 5.2.4; Table 5.2.1) with a total of 20 measures taken and the mean values calculated.

**Figure 5.2.4.** Representative M-mode echocardiogram and respective measurements taken over a heartbeat in a 14 day seizure animal
Table 5.2.1. Measurements of left ventricular function using echocardiography

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Units</th>
<th>Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV internal dimension, diastolic and systolic (LVIDd, LVIDs)</td>
<td>mm</td>
<td>-</td>
</tr>
<tr>
<td>LV posterior wall thickness, diastolic and systolic (LVPW)</td>
<td>mm</td>
<td>-</td>
</tr>
<tr>
<td>Interventricular septal, diastolic and systolic (IVSd and IVSs)</td>
<td>mm</td>
<td>-</td>
</tr>
<tr>
<td>End volume, diastolic and systolic (EDV and ESV)</td>
<td>ml</td>
<td>-</td>
</tr>
<tr>
<td>Ejection fraction (EF)</td>
<td>%</td>
<td>EF = SV/EDV × 100</td>
</tr>
<tr>
<td>Fractional shortening (FS)</td>
<td>%</td>
<td>FS = (LVIDd-LVIDd)/LVIDd × 100</td>
</tr>
<tr>
<td>Stroke volume (SV)</td>
<td>ml</td>
<td>SV = EDV - ESV</td>
</tr>
</tbody>
</table>

5.2.7. Arrhythmia risk.

7 day transmitter rats. Following the 7 day behavioural study, rats (n=5/group) were administered a bolus injection of the arrhythmogenic agent, aconitine (2.5 mg/kg sc, determined by trialling doses) during ECG monitoring in order to assess vulnerability to arrhythmias. The latency from aconitine administration to presentation of arrhythmias (first premature ventricular contraction, bigeminy, salvo, ventricular tachycardia and ventricular fibrillation) were recorded in each animal (Table 5.2.2 and Figure 5.3.13). The rats were sacrificed at presentation of ventricular fibrillation as required by the University Animal Ethics Committee.

14 and 28 day cannula rats. Rats were sedated with domitor and a local anaesthetic, lignocaine (2 mg/kg sc) applied to allow ECG electrode placement. Two small incisions (1 cm), were made to the chest to allow ECG electrode placement on the xiphoid process and into the right anterior media-stinum. Baseline ECG recordings (5 minutes) were taken immediately prior to aconitine (2.5 mg/kg sc) administration and arrhythmia risk assessed as described above.

Table 5.2.2. Classes according to the Lambeth Conventions (Walker et al., 1988; Grippo et al., 2004)

<table>
<thead>
<tr>
<th>Convention</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Premature ventricular contraction (PVC)</td>
<td>Premature QRS with no P wave</td>
</tr>
<tr>
<td>Bigeminy</td>
<td>Two or more PVC, characterised by a minimum sequence: P, QRS, PVC, P, QRS, PVC.</td>
</tr>
<tr>
<td>Salvo</td>
<td>Two or three PVCs for each P wave and corresponding QRS complex</td>
</tr>
<tr>
<td>Ventricular tachycardia</td>
<td>A series of four PVCs for each P wave and corresponding QRS complex</td>
</tr>
<tr>
<td>Ventricular fibrillation</td>
<td>No discernible rhythm</td>
</tr>
</tbody>
</table>

5.2.8. Histology and immunohistochemistry. At the end of the study, the rats were anaesthetised with halothane using an induction chamber and hearts rapidly excised as described in Chapter 2.2.8. Rats were decapitated and each entire brain placed into a mould and suspended in CRYO-OCT Compound (Tissue-Tek, USA). The tissue block was gently frozen in an n-hexane bath immersed in liquid nitrogen. Tissue was stored at -80 ºC until sectioning. The brain was sliced at -20ºC into 16 μm thick sections using a 101 Leica CM 1850 cryostat (Meyer Instruments Inc., TX, USA).
Every fifth section of the hippocampus was then mounted onto Flex IHC microscope slides (Dako, Denmark).

5.2.8.1. Cardiac apoptotic cell death (ApopTag®). DNA strand breaks were assessed enzymatically using an ApopTag® Peroxidase (Millpore, Darmstadt, Germany) kit to detect the presence of free 3'-OH termini (Darzynkiewicz et al., 2008). Paraffin embedded ventricular sections were washed in xylene and rehydrated with sequentially decreasing concentrations of ethanol (100%, 95%, 85%, 50%). Sections were then treated with proteinase K (1:2500) for 20 minutes and endogenous peroxidases blocked with hydrogen peroxide (3%) for 5 minutes. Following a phosphate buffered saline (PBS, pH 7.4) wash, sections were incubated with equilibration buffer (75 μL/5 cm²) for 15 minutes in a humidified chamber at room temperature. Sections were then incubated according to the kit instructions with working strength terminal deoxynucleotidyl transferase (TdT) enzyme (55 μL/5 cm²) for 60 minutes in a humidified chamber at 37°C. The reaction was terminated by incubation in the pre-warmed stop-wash buffer provided (30 minutes; 37°C). Sections were then washed in PBS before anti-digoxigenin conjugate (65 μL/5 cm²) was applied to each section and allowed to incubate for 30 minutes at room temperature. Nuclei undergoing apoptosis were visualised using a 3,3-diaminobenzidine (DAB; Vector Laboratories, USA) kit which stains for peroxidase. A DAB solution consisting of a stock solution of substrate reagent (pH 7.5, 0.04 ml), DAB (0.1 ml) and hydrogen peroxide (0.04 ml) in 5.0 ml of distilled water, was applied to the slides, under low light conditions and incubated for 10 minutes. The tissue was counterstained with Gills #2 Haematoxylin for 10 seconds and the sections gently washed with tap water. The slide were dehydrated in ascending concentrations of ethanol (50%-100%) for 3 minutes each and cleared in xylene for 30 seconds. Finally, tissue sections were air-dried and coverslipped using Di-N-butylphthalate (DPX in xylene).

5.2.8.2. Cardiac macrophage infiltration (CD68). Tissue sections were deparaffinised with xylene and rehydrated using serial ethanol concentrations (50-100%). Sections were antigen retrieved using sodium citrate buffer (10 mM, pH 6) for 20 minutes at 95°C and allowed to cool at room temperature for 20 minutes. Tissues were blocked with animal free blocker (1:5) for 2 hours and then washed with PBS containing 0.1% bovine serum albumin (BSA). Sections were then incubated overnight at 4°C with mouse anti-CD68 monoclonal IgG anti-rat antibody (1:100; Abcam) in PBS containing BSA (0.1%). Slides were washed in PBS and then blocked with peroxidase (0.3% H₂O₂) for 10 minutes. Sections were washed and incubated at room temperature for 90 minutes with the secondary peroxidase goat anti-mouse polyclonal IgG antibody (1:250). The tissue was stained (10 minutes) using the DAB substrate kit (as described above and counter
stained with haematoxylin. The sections were finally rehydrated with ethanol and a coverslip secured with DPX.

5.2.8.3. **Hippocampal apoptotic cell death (ApopTag®).** Brain tissue sections were thawed and fixed in NBF (10%) for 5 minutes. The tissue was then treated with ethanol:acetic acid (2:1) for 10 minutes at -20°C prior to proteinase K (1:2500). The Apoptag® peroxidase procedure was then performed as described above.

5.3.8.4. **Hippocampal Macrophage infiltration (CD11).** Whole brain tissue sections were thawed and fixed in NBF (10%), blocked for 2 hours with animal free blocker (1:5 dilution in distilled water) and washed with 1% BSA to block non-specific antibody binding. Sections were then incubated overnight at 4°C with mouse anti-rat monoclonal antibody to the microglial marker CD11 (1:400). Sections were then incubated with HRP conjugated goat anti-mouse in 1% BSA/PBS and incubated at room temperature for 30 minutes. Slides were washed with PBS containing 0.3% Triton X-100 between steps. DAB substrate kit (6 minutes) was used to stain for the secondary antibody and co-stained with haematoxylin. Sections were washed in tap water and dehydrated with serial ethanol concentrations (50-100%) and xylene and cover-slipped using DPX.

5.3.8.5. **Analysis and quantification of histology and immunohistochemistry:** Examination of the above stained sections, including MSB stained ventricular sections prepared as described in Chapter 2.2.8, was conducted using an Aperio ImageScope (sections were blinded to the observer). The extent of positive (blue) pixels or negative (white) were quantified across all three layers using ImageScope v.11 software. ImageScope IHC Nuclei software was used to determine the number of apoptotic cells in both the heart and hippocampus and expressed as a ratio of the total stained tissue area. Apoptotic cardiomyocytes were counted and confirmed visually based on morphological appearance. Ventricular CD68 was quantified by counting the number of cells with CD68 positive staining using ImageScope IHC membrane software. Apoptosis and CD68 were presented as the number of cells per area (mm²). Hippocampal CD11 positive cells showed diffuse staining throughout the hippocampus and due to the spindly shape of the activated microglia were difficult to quantify cell numbers; therefore the percentage of positive stained (brown) pixels were determined using Photoshop.

5.2.9. **Statistics.** Statistical analysis was performed using Prism™ v.6 (GraphPad, San Diego, USA). Behavioural data were analysed using a Kruskal-Wallis test with Bonferroni post-hoc. EEG, ECG, BP and HRV variables were analysed using a 2-way repeated measures AVOVA with Bonferroni post-hoc analysis. Vagal sympathetic effect, noradrenaline and cortisol levels were
analysed with a 1-way repeated measures ANOVA comparing to baseline line. Statistical significance was determined as $P<0.05$. Data presented as mean $\pm$ SEM.
5.3. Results

5.3.1. Seizure Activity

Behavioural scores remained constant throughout the study in the control group. Intrahippocampal injections of saline had no effect on rat behaviours or EEG activity (Figure 5.3.1). KA administration resulted in an immediate increase in seizure activity which remained significantly elevated (mean seizure behaviour of 2.9 ± 0.1 compared to 0.1 ± 0.03 in the controls) over the 180 minute period. KA dosed animals had an elevated cumulative behavioural score of 530 ± 19, with 326 ± 64 WDS recorded (Table 5.3.1). These seizure behaviours were associated with significant increases in EEG activity across all frequency bands (Figure 5.3.1A) with the greatest increase observed within the initial 60 minutes (maximum increases of 27- to 42-fold across the delta to beta frequencies).

<table>
<thead>
<tr>
<th>Table 5.3.1. Behavioural activity following KA seizures</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Cumulative</td>
</tr>
<tr>
<td>WDS</td>
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<tr>
<td>Level 4</td>
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<td>Level 5</td>
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*Behavioural score following intrahippocampal saline or KA administration over 180 min. The cumulative score was the sum of the maximum behaviour every minute. Values shown represent the total number of wet dog shakes, Level 4 and Level 5 behaviours observed. *P<0.05 compared to control.*

5.3.2. ECG Analysis

Intrahippocampal saline administration in the control animals had no significant effect on HR QTc, T wave amplitude or systolic BP (mean of 354 ± 10 b.p.m., 43 ± 3 ms, 92 ± 5m, 98 ± 3 mmHg, respectively). KA administration resulted in a significant maintained increase in mean HR by 20% which persisted for the 180 minute study (Figure 5.3.3). This was associated with prolongation of the QTc interval by 10-15% over 76-178 minutes and T wave elevation (maximum of 48% at 94 minutes) at 140-176 minutes (Figure 5.3.4 and 5). All ECG variables had returned to baseline by the 24 hour recording period. Seizure activity also resulted in an increase (13-25%) in BP 1 to 48 hours post-KA and at 14 days (Figure 5.3.6). Figure 5.3.2 represents a continuous EEG and HR recording throughout the night until 4 am, demonstrating seizure-related EEG spiking occurring throughout the night coinciding with increases in HR (465 b.p.m. at 11 pm). There were no significant changes in the HRV (SDNN, LF or HF) following saline or KA administration (data not shown), however there was a significant increase in the LF/HF ratio by 1.9- and 1.8-fold 48 hours and 7 days post-KA, respectively (Figure 5.3.7).
**Figure 5.3.1.** EEG (A) and behavioural (B) changes following saline or KA administration. Histograms represent normalised power spectral density (PSD; mean ± SEM) for each 10 min bin across the delta (1.25-4.5 Hz), theta (4.75-6.75 Hz), alpha (7.0-12.5 Hz) and beta (12.75-35.0 Hz) frequency bands. Mean behavioural score (mean ± SEM) every 5 min. *P<0.05 compared to control.

**Figure 5.3.2.** Represents an individual trace of EEG activity activity (gray trace) and heart rate (black trace) over 18 hours following intrahippocampal KA administration.
Figure 5.3.3. Effect of saline or KA on heart rate (beats per minute, b.p.m.) in rats. Data was analysed every 2 minutes over 180 min with a 30 min period at 24 and 48 hours and 7 days (mean ± SEM). *P<0.05 compared to control.

Figure 5.3.4. Effect of saline or KA on the QTc interval in rats. Data was analysed every 2 minutes over 180 min with a 30 min period at 24 and 48 hours and 7 days (mean ± SEM). *P<0.05 compared to control.

Figure 5.3.5. Effect of saline or KA on the T wave amplitude in rats. Data was analysed every 2 minutes over 180 min with a 30 min period at 24 and 48 hours and 7 days. Data was normalised to the baseline T wave amplitude and presented as mean ± SEM. *P<0.05 compared to control.
Figure 5.3.6. Effect of saline or KA on systolic blood pressure in rats. Presented as mean ± SEM. #P<0.05 compared to baseline, *P<0.05 compared to control.

Figure 5.3.7. The effect of saline (black) or KA (white) administration on frequency analysis of heart rate variability. Data was analysed over a 5 min period throughout the study and presented as a low frequency/high frequency (LF/HF) ratio. Data mean ± SEM. #P<0.05 compared to baseline.
5.3.3. Vagal-sympathetic effect

Ipratropium administration at the pre-seizure recording period resulted in an increase in HR by 103 ± 8 b.p.m. while atenolol decreased HR by 40 ± 8 b.p.m. (Figure 5.3.8A and B). Ipratropium administration at 48 hours post-seizure produced a significantly attenuated response compared to pre-treatment (increase of 54 ± 15 b.p.m.); this was associated with a greater drop in HR by 79 ± 9 b.p.m. following atenolol administration. During the pre-seizure recording combined ipratropium/atenolol administration revealed an intrinsic HR of 362 ± 3 b.p.m. (pre-treatment HR of 348 ± 9 b.p.m.) and a vagal-sympathetic effect of 1.04 ± 0.02. These were significantly reduced at the 48 hour and 7 day recording period, where the VSE dropped to 0.87-0.89.

![Figure 5.3.8](image)

**Figure 5.3.8.** Vagal and sympathetic balance on heart rate 48 hours and 7 days following seizure induction. **A)** Change in heart rate during the 20 min following ipratropium (vagal effect) or atenolol (sympathetic effect) administration. **B)** The histogram shows the baseline and intrinsic (drug denervated) HR, while the line graph demonstrates the vagal sympathetic effect (VSE; iHR/HR ratio). *P<0.05 compared to pre-seizure.
5.3.4. Noradrenaline and Cortisol Plasma Levels

Prior to seizure induction, baseline noradrenaline levels were 0.28 ± 0.12 ng/ml and cortisol was 0.04 ± 0.006 ng/ml (Figure 5.3.9). KA administration resulted in a significant increase in plasma noradrenaline levels by 8.9-fold at 3 hours following seizure induction, peaking at 48 hours (plasma noradrenaline of 2.54 ± 1.03 ng/ml). This peak at 48 hours was also associated with an elevation in cortisol levels by 2.5-fold to 0.6 ± 0.18 ng/ml. Interestingly, noradrenaline levels were still elevated by 5-fold (P<0.06 compared to baseline) at 28 days.

![Graph A: Plasma Noradrenaline Levels](image)

![Graph B: Plasma Cortisol Levels](image)

**Figure 5.3.9.** The effect of seizure activity on plasma noradrenaline (NA; A) and cortisol (B) in rats over the course of the study. *P<0.05 compared to baseline.

5.3.5 Echocardiogram

Left ventricular function was assessed via echocardiogram in sedated rats (Figure 5.3.10 and 11). There was no significant change in left ventricular function in the control animals at 7 and 14 days. In the seizure animals, there was a significant increase in left ventricular internal diameter during systole at 14 and 28 days which was associated with a significant increase in end systolic volume by 3.3-fold (Figure 5.3.10A and D). All KA treated groups had a reduction in the left ventricular posterior wall thickness during systole compared to controls. In the seizure hearts, there was a significant reduction in ejection fraction by 11-16% at all time points, which was associated with a drop in fractional shortening by 30% and 23% at 14 and 28 days, respectively (Figure 5.3.11).
Figure 5.3.10. Left ventricular (LV) function following saline or KA administration at 7, 14 and 28 days. LV internal dimension (LVID), LV posterior wall thickness (LVPW), interventricular septum (IVS) and end volume (EV) were measured (mean ± SEM). *P<0.05 compared to control.

Figure 5.3.11. Left ventricular function following saline or KA administration at 7, 14 and 28 days. Ejection fraction (EF), fractional shortening (FS) and stroke volume (SV) were measured using echocardiography (mean ± SEM). *P<0.05 compared to control.
5.3.6. Arrhythmia risk

In conscious control rats, aconitine administration resulted in the development of arrhythmias within 40 minutes with premature ventricular contractions (PVC), bigeminy and eventually ventricular fibrillation observed at 32 ± 4.9, 34 ± 3.2 and 46 ± 4.3 minutes, respectively (Figure 5.3.12). At 7 days post-KA animals had an increased susceptibility to benign arrhythmias with PVC and bigeminy occurring 55% and 29% earlier than controls. Seizure rats had a reduced latency to potentially fatal arrhythmias such as ventricular tachycardia and fibrillation compared to control animals. Anaesthetised rats took longer to develop arrhythmias compared to the conscious free moving 7 day rats. Animals which were at 28 days post-KA developed PVC at 37 ± 8.8 minutes which was 39% earlier than controls. At 14 and 28 days, seizure animals had increased susceptibility to salvo, ventricular tachycardia and ventricular fibrillation.

Figure 5.3.12. Shows the effect of aconitine on latency to arrhythmias in saline and KA treated rats. The 7 days rats were concious and free-moving, while the 14 and 28 days rats were anethetised prior to aconitine administration. *P<0.05 compared to control.
Chapter 5: Cardiac dysfunction following intrahippocampal KA-induced seizures

Figure 5.3.13. The effect of saline or kainic acid on cardiac structure. (A) Percentage of tissue positively stained for fibrosis. (B) Percentage of clear area used as a way of determining the presence of oedema. (C) The total number of apoptotic (ApopTag) and (D) macrophage (CD68) cells in the heart. *P<0.05 compared to control.

5.3.7. Histology and Immunohistochemistry

MSB staining of the hearts at 7, 14 and 28 days showed significant evidence of fibrosis (4.6-4.7% of the left ventricular area; Figure 5.3.15A) and reversible ischaemic damage as demonstrated by myocyte vacuolisation (Figure 5.3.15C and 16C). There was a significant increase in oedema 22.3 ± 3.0% at 7 days which was reduced in the 14 and 28 day rats (Figure 5.3.13B). MSB staining revealed evidence of inflammatory cell infiltration which was supported by positive CD68 cells (increased by 3.4- to 4.6-fold, Figure 5.3.13D) demonstrating macrophage presence. Figure 5.3.16 demonstrates two micro-lesions with evidence of necrosis, fibrosis and inflammatory cell infiltration, including macrophages. Cardiac immunohistochemistry demonstrated that seizure activity resulted in an increase in the extent of ApopTag positive cells by 2.9- to 4-fold at 7 to 28 days following seizure insult (Figure 5.3.13C and 14). Apoptotic cardiomyocytes were randomly scattered throughout the myocardium with some ApopTag positive cells observed perivascularly and within micro-lesions, however morphology resembled inflammatory cells. Hippocampal tissue in seizure rats, showed significant evidence of macrophage infiltration and apoptosis in the hippocampus. There was a 2-fold increase in ApopTag positive cells in the ipsilateral hippocampus at 7 days compared to control (6 ± 1.2%, Figure 5.3.17). There was significantly more CD11 positive staining in the ipsilateral (1-2.2%) hippocampus compared to the contralateral side (2.5-4%, Figure 5.3.18).
Chapter 5: Cardiac dysfunction following intrahippocampal KA-induced seizures

Figure 5.3.14. Micrographs of apoptotic cells (arrows) in the left ventricular myocardium following seizure induction. ApopTag positive myocytes were randomly dispersed throughout the myocardium (A-B), with ApopTag positive staining suggestive of apoptotic inflammatory cells around vessels (C) and in microinfarcts (D).

Figure 5.3.15. Micrographs of MSB stained myocardium in control (A and B) and seizure (C and D) animals. Control hearts had normal morphology while seizure hearts had evidence of myocyte vacuolisation (C, arrows), oedema (C) and fibrotic deposition (D).
Figure 5.3.16. Micrographs of the left ventricular myocardium (A and C; MSB stained). These images represent the presence of two micro-lesions in kainic acid treated animals at 7 days. Perivascular fibrosis (A) with macrophage infiltration (B). Intersitial fibrosis (C) with macrophage infiltration (D). Myocyte vacuolisation demonstrated with arrows (A and C).


Figure 5.3.17. Representative micrograph of the hippocampus in a control (A) and a kainic acid 7 days (B) rat following immunohistochemistry staining for apoptosis (ApopTag). *P<0.05 compared to ipsilateral, *P<0.05 compared to control.
Figure 5.3.18. Representative micrograph of the hippocampus in a control (A) and a kainic acid 7 days (B) rat following immunohistochemistry staining for macrophage infiltration (CD11). *P<0.05 compared to ipsilateral, *P<0.05 compared to control.
5.4. Discussion

This study clearly demonstrates that seizures result in a deterioration in cardiac function due to elevated sympathetic activation. Intrahippocampal KA produced sustained seizure activity lasting for several hours, similar to previous studies (Bouilleret et al., 1999; Raedt et al., 2008). Seizure activity was associated with early activation of the sympathetic system, as demonstrated by increased plasma noradrenaline, tachycardia and elevated BP within 3 hours. Hearts from seizure animals had evidence of dilated cardiomyopathy as determined by increased left ventricular dimensions and decreased ejection fraction. This deterioration may be due to the development of micro-lesions and fibrotic deposition increasing susceptibility to aconitine-induced arrhythmias, which did not reduce during the study period.

Generalised seizures have been associated with cardiac dysfunction in animal studies. In piglets, pentylenetetrazole-induced tonic-clonic seizures coincided with increases in HR, BP, ventilation and metabolic acidosis (Terndrup et al., 1994). The same animal model produced a fall in cardiac output by 30-60% during recurrent seizures in 63% of animals, which was accompanied by a transient drop in HR and an increase in BP (Kreisman et al., 1993). These results are supported by studies conducted in rats which showed that pilocarpine-induced SE produced an increase in HR and BP (~510 b.p.m. and ~150 mmHg, respectively), within 60 minutes of SE (Metcalf et al., 2009a). These rats had an increased susceptibility to aconitine induced arrhythmias with ventricular tachycardia occurring nearly 40% faster than control hearts. Metcalf et al. (2009b) also looked at the effect of VSE in SE rats and found that the VSE (iHR/HR) dropped from 0.98 to 0.87 at 7 days, suggesting sympathetic dominance. This was also supported by Damasceno (2013) where audiogenic seizures, resulted in a VSE of 0.89, although disappointingly no timeline was given (Damasceno et al., 2013). This chapter reported an increase in noradrenaline levels which is supported clinically where SE is associated with elevated adrenaline and noradrenaline levels (Benowitz et al., 1986).

There is very little, previously published data on the effect of seizure on cardiac function as measured by echocardiogram. A paper by Sakamoto et al. (2008) did indicate that KA decreased ejection fraction and increased left ventricular diastolic diameter in a subset of rats, but failed to deliver any information on incidence, extent or time course of left ventricular dysfunction. The present study, provides evidence of dilated cardiomyopathy, as determined by an increase in LV left ventricular dimension, a reduction in wall thickness and increased blood volumes (SV and end diastolic and systolic volumes) in seizure animals. These hearts had reduced fractional shortening, most likely as a consequence of fibrotic deposition leading to a stiffening of the
myocardium, contributing to a decrease in ejection fraction (Table 5.1). Dilated cardiomyopathy is the most common cardiomyopathy and is characterised by an increase in myocardial volume and ventricular wall thinning with reduced cardiac contractility. Dilated cardiomyopathy is commonly reported clinically in young-middle aged males (20-50 years; Fatkin et al., 1999; McNamara et al., 2001) and may contribute to the cardiomyopathy reported in seizure patients of this age group. Patients with dilated cardiomyopathy have an estimated 5 year mortality of 50% as patients generally develop heart failure and complications such as arrhythmia or thromboembolism (Grogan et al., 1995). Dilated cardiomyopathy has a strong genetic link (20-35%), although it can also occur as a consequence of elevated catecholamine levels, occurring in phaeochromocytoma, tachycardia and ischaemic cardiac damage (Hershberger et al., 2010). Reduced fractional shortening, decreased ejection fraction and an increase in left ventricular dimensions are required for diagnosis of dilated cardiomyopathy (Luk et al., 2009). Patients may also present with tachycardia, changes in blood pressure and ECG changes. The progressive increase in left ventricular dimension can lead to wall thinning with fibrosis worsening contractility, which is similar to the cardiomyopathy presented in the current study (Table 5.4.1).

The pathology reported in the present study, is also similar to features observed in tachycardia- and stress-induced cardiomyopathies (Table 5.4.1). Tachycardia-induced cardiomyopathy is characterised by atrial or ventricular myocardial dysfunction resulting from increased contraction rates with no underlying structural heart disease (Khasnis et al., 2005). Elevated sympathetic stimulation and catecholamine levels produce left ventricular dilation, elevated end-diastolic pressure, decreased wall thickness and reduced ejection fraction (Khasnis et al., 2005). Stress-induced cardiomyopathy (also called Takotsubo syndrome) is a reversible pathology recognised by apical ventricular ballooning and reduced ejection fraction occurring consequent to a stressful event (emotional or physical) enhancing sympathetic stimulation (Virani et al., 2007; Parodi et al., 2007; Looi et al., 2012b; Looi et al., 2012a). Stress-induced cardiomyopathy is more common in elderly females and is generally reversible, however it is associated with a mortality rate of 16% (Lee et al., 2010). Dib and co-authors (2009) reported that stress-induced cardiomyopathy was associated with reduced ejection fraction at the time of admission, although it returned to normal within 5-7 days. Pertinently stress-induced cardiomyopathy has been linked to epilepsy with 1.8% of patients presenting with seizures (Le et al., 2011a; Schneider et al., 2010; Rodriguez de Antonio et al., 2011; Naganuma et al., 2011; Stollberger et al., 2011; Le et al., 2011b; Hassan, 2011; Porto et al., 2013).
### Table 5.4.1. Comparison of current study indices against defined features in dilated, tachycardia- and stress-induced cardiomyopathies

<table>
<thead>
<tr>
<th></th>
<th>Current seizure study</th>
<th>Dilated cardiomyopathy</th>
<th>Tachycardia-induced cardiomyopathy</th>
<th>Stress-induced cardiomyopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart Rate</strong></td>
<td>Increased</td>
<td>May be increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>QTc Interval</strong></td>
<td>Prolonged</td>
<td>-</td>
<td>Prolongation</td>
<td>Prolongation</td>
</tr>
<tr>
<td><strong>T wave elevation</strong></td>
<td>Increased</td>
<td>May have ST changes</td>
<td>-</td>
<td>ST elevation</td>
</tr>
<tr>
<td><strong>Blood pressure</strong></td>
<td>Increased</td>
<td>Increased systolic</td>
<td>Decreased or no change in MAP</td>
<td>Increased BP</td>
</tr>
<tr>
<td><strong>HRV</strong></td>
<td>Increased LF/HF ratio (after 48 hr)</td>
<td>-</td>
<td></td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Catecholamine</strong></td>
<td>Increased plasma (spill over)</td>
<td>-</td>
<td>Reduced cardiac noradrenaline uptake</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Cortisol</strong></td>
<td>Increased (at 48 hr)</td>
<td>-</td>
<td>-</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Cardiac enzymes</strong></td>
<td>Increased at 24 hr</td>
<td>-</td>
<td>-</td>
<td>Mild to moderate elevated up to 24 hr</td>
</tr>
<tr>
<td><strong>Echo: LVID</strong></td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>: LVPW</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased or increased</td>
<td>Decreased</td>
</tr>
<tr>
<td>: IVS</td>
<td>Decreased</td>
<td>-</td>
<td>-</td>
<td>Decreased</td>
</tr>
<tr>
<td>: EF</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>: FS</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td><strong>Arrhythmias</strong></td>
<td>Increased susceptibility</td>
<td>Arrhythmias (VT) and sudden death reported</td>
<td>Arrhythmias (VT) and sudden death reported</td>
<td>Ventricular tachycardia</td>
</tr>
<tr>
<td><strong>Histology:</strong></td>
<td>Apoptosis</td>
<td>Areas of myocyte death may be present</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Myocyte vacuolisation</td>
<td>Myocyte vacuolisation may occur</td>
<td>-</td>
<td>Myocyte vacuolisation</td>
</tr>
<tr>
<td></td>
<td>HBN</td>
<td>-</td>
<td>-</td>
<td>HBN in some cases</td>
</tr>
<tr>
<td></td>
<td>Interstitial and perivascular fibrosis</td>
<td>Interstitial fibrosis</td>
<td>Sub-endocardial fibrosis</td>
<td>Interstitial and perivascular fibrosis</td>
</tr>
<tr>
<td></td>
<td>Inflammatory cell infiltration</td>
<td>May have leukocyte infiltration</td>
<td>Inflammation</td>
<td>Leukocyte infiltration</td>
</tr>
</tbody>
</table>

*No reported alteration (-). BP: blood pressure, Echo: echocardiography, EF: ejection fraction, FS: Fractional shortening, HBN: hypercontracture band necrosis, HRV: heart rate variability, LVID: left ventricular internal dimension, LVPW: left ventricular posterior wall thickness, IVS: interventricular septum, MAP: mean arterial pressure, VT: ventricular tachycardia (Khasnis et al., 2005; Lyk et al., 2009; Hershberger et al., 2010; Porto et al., 2013).*
Clinically, generalised seizure activity has been associated with elevated troponin I levels but with normal echocardiography (Brobbey & Ravakhah, 2004; Hajsadeghi et al., 2009; Eskandarian et al., 2011; Akashi et al., 2012). As these echocardiograms were taken at the time of seizure presentation, cardiomyopathy may not have developed to the point where deterioration in left ventricular function was detectable. The elevated troponin I levels suggest that there is structural cardiac injury occurring and this may accumulate with seizure incidence. A case study, reported a 64 year old female presenting with generalised tonic-clonic seizures and elevated systolic BP and HR, with left ventricular dilation leading to a diagnosis of stress-induced cardiomyopathy. Left ventricular dilation was consequently reversed at follow up at 12 and 14 weeks (Weeks et al., 2007). Tigaran et al. (2003) also reported echocardiographic abnormalities in 9% of refractory epilepsy cases, with evidence of posterior wall hypertrophy and trivial aortic regurgitation.

The use of intrahippocampal KA in this chapter, confirms that seizures result in cardiac damage which is not a direct consequence of KA. This study demonstrated that seizures result in dilated cardiomyopathy as a consequence of sympathetic hyperactivation producing tachycardia-induced ischaemic damage. Sustained tachycardia increases cardiac oxygen demand while reducing oxygen supply, this in combination with catecholamine-induced vasospasm results in micro-infarcts and deterioration in left ventricular dysfunction. It is important that this sympathetic activity is attenuated by pharmacological intervention and this will be further investigated in Chapter 6.
Chapter 6

Therapeutic interventions in seizure-induced cardiomyopathy
6.1. Introduction

Chapter 5 demonstrated that seizures are associated with increased sympathetic activity resulting in dilated cardiomyopathy. This suggests that it is important to prevent the elevation in HR during the acute phase of the seizure. Chapter 4 and Appendix 8.3 demonstrated that atenolol is an effective prophylactic therapy in SE and generalised seizures.

Randomised controlled trials in SE have provided evidence of a high (85%) success rate with benzodiazepines. This has led to the use of diazepam or lorazepam as first-line drug interventions in patients with SE (Lowenstein & Alldredge, 1998; Sirven & Waterhouse, 2003; Mehta et al., 2007). Diazepam potentiates the inhibitory effect of GABA at GABA_A receptors, to increase the Cl^- current, thereby increasing the threshold of neuronal firing (Meldrum, 1996). GABA_A receptors are heterogeneous pentameric receptors, made up of α_1-6, β_1-4, γ_1-3 and δ subunits (Abraham et al., 2000). The benzodiazepine (BZP) binding site straddles the α and γ subunits to allosterically enhances the action of GABA on the Cl^- pore (Figure 6.1.1). Diazepam has also been found to protect hippocampal and striatal neurons following transient cerebral ischaemia (Schwartz et al., 1994; Schwartz et al., 1995) and consequently may attenuate the hippocampal damage reported in Chapter 5 in KA-treated rats. Conversely, clinical use of diazepam has not been indicated to alter HR but protects against SE-induced arrhythmias (Alldredge et al., 2001; Nascimento et al., 2007). This chapter examines whether preventing seizure progression with diazepam reduces the extent of seizure-induced cardiomyopathy.

This study also further examines the hypothesis that combination intervention with atenolol produces additional cardiac protection.

![GABA Receptor](image)

**Figure 6.1.1.** GABA_A receptor conformation with α, β and γ subunits. GABA and benzodiazepine (BZP) binding sites. Adapted from Del Favero and Fringuello (2013).
6.2 Methods

6.2.1 Materials. All reagents were purchased from BDH (Palmerston North, New Zealand) and Sigma-Aldrich (Auckland, New Zealand). Prescription remedies and diazepam were obtained from the University of Otago’s Drug Control Officer at the University of Otago Animal Welfare Office. KA was purchased from Tocris (Bristol, UK) and dissolved in saline (0.9% NaCl). Atenolol and aconitine were purchased from Sigma-Aldrich (Auckland, New Zealand).

6.2.2. Animals. Sprague-Dawley rats (80 males; 320-350g) were obtained from the University of Otago Animal Resource Unit. The animals were housed on a 12 hour light/dark cycle at 22°C with food and water ad libitum and left to acclimatise for 5 days prior to surgery. Experiments were performed in accordance with the regulations of the University of Otago’s Committee on Ethics in the Care and Use of Laboratory Animals and the “Use of Laboratory Animals (NIH Publication No. 85-23, 1996)”.

6.2.3. Experimental Protocol. All animals were instrumented with an intrahippocampal drug cannula secured into the right hippocampus as described in Chapter 5.2.3. A subset of rats ($n=40$ rats) were also implanted with an EEG/ECG transmitter (as detailed in Chapter 2.2.4). Animals were randomly allocated to saline, diazepam (5 mg/kg, sc, as determined in Appendix 8.6), atenolol (5 mg/kg, sc) or diazepam+atenolol treatment groups which was administered 60 minutes post-KA ($n=20$/group). Diazepam (1 mg/kg, sc, bid) and atenolol (5 mg/kg, sc, once daily) were administered for the remainder of the study. The observer was blinded to the allocated drug treatment and blinding was maintained until all data was analysed. The telemetered rats were euthanised at 7 days, while the cannula rats were continued to 14 days (Figure 6.2.1), at which time the hearts and brains were removed for histology as described in Chapter 5.3.7.

Figure 6.2.1. Delegation of rats into respective, experimental studies and time courses.
6.2.4. **Seizure induction.** Seizures were induced by an intrahippocampal infusion of KA (2 nmol, 1 μl given over 1 minute) and all animals were monitored for 3 hours as described in Chapter 5.2.4. EEG/ECG activity was recorded in the telemetered rats with behavioural activity recorded every 15 sec (as described in Chapter 2.2.5). The cannulated rats were monitored over 3 hours to ensure that successful seizure-induction of Level 4 behaviours. In a subset of animals \((n=6/\text{group})\), arterial systolic BPs were recorded prior to seizure induction and at 15, 30, 60 and 180 minutes and at 7 and 14 days post-KA. Four repeat blood pressure recordings were taken using LabChart and the mean of the systolic blood pressure values determined. In another subset of animals, tail vein blood samples (0.5 mL, \(n=4/\text{group}\)) were taken over the course of the study to determine troponin levels.

6.2.5. **Troponin I levels.** Blood was centrifuged at 3000 r.p.m. for 3 minutes and plasma frozen (-80°C) until use. Troponin I levels were determined using a high sensitivity rat cardiac troponin I ELISA kit (Life Diagnostics, USA, 2010-2-HSP). Rat plasma (100 μl) was added in duplicate to the 96 well plate with 100 μl cTnI HRP conjugate for 60 minutes at room temperature. Wells were washed and 100 μl of TMB Regent to each well and left to incubate for 20 minutes at room temperature. The reaction was stopped using the proprietary stop solution and the absorbance was immediately read (450 nm).

6.2.6. **Echocardiography.** Rats (\(n=5/\text{group}\)) were sedated with domitor and left ventricular dimensional and functional parameters were measured using the Vivid E9 ultrasound system (GE Healthcare) as described in Chapter 5.2.6.

6.2.7. **Arrhythmia risk.** Latency to arrhythmia induction was assessed as described in Chapter 5.2.7 using aconitine (2.5 mg/kg, sc). The 14 day cannula rats were anaesthetised and ECG electrodes implanted as described prior to aconitine administration. ECG waveforms were monitored to assess latency to arrhythmias. The duration from aconitine administration to first premature ventricular contraction, bigeminy, salvo, ventricular tachycardia, ventricular fibrillation, were recorded in each rat. Animals were terminated with presentation of ventricular fibrillation as required by the University Animal Ethics Committee.

6.2.8. **Histology and Immunohistochemistry.** At the end of the study, the rats were anaesthetised with halothane using an induction chamber and hearts rapidly excised as described in Chapter 2.2.8. Rats were decapitated and each entire brain placed into a mould and suspended in CRYO-OCT Compound (Tissue-Tek, USA). The brain was sliced at -20°C into 16 μm thick sections (as described in Chapter 5.2.8) and every fifth section of the hippocampus studied. Left ventricular section were stained with MSB and the extent of fibrotic deposition was quantified (as describe
in Chapter 5.3.8.5). Immunohistochemistry (ApopTag, CD11 and CD68) of heart and brain slice were performed and analysed blinded as described in Chapter 5.2.8.

6.2.9. Data analysis and statistics. EEG data was analysed, as described in Chapter 2.2.6, using Fast Fourier transformation (FFT) to quantify the frequency bands and normalised to baseline. The cumulative score was determined as the sum of the maximum score every minute over the 3 hour recording period. The total number of WDS, Level 4 and Level 5 behaviours were quantified over the 180 minute recording period. ECG data was analysed using LabChart6 Pro ECG Analysis module software to assess heart rate (HR), QTc intervals (as described in Chapter 2.2.7) and T wave amplitudes. Data were analysed every two minutes in one minute blocks over the 3 hour observation period. HRV was analysed every a 5 minutes period throughout the study, as described in Chapter 3.2.4. Statistical analysis was performed using Prism™ v.6 (GraphPad, San Diego, USA). Behavioural data were analysed using a Kruskal-Wallis test with Bonferroni post-hoc. EEG, ECG, BP and HRV variables were analysed using a 2-way repeated measures AVOVA with Bonferroni post-hoc analysis. Statistical significance was determined as $P<0.05$. Data are presented as mean ± standard error of the mean (SEM).
6.3. Results

6.3.1 Seizure activity

In all animals KA administration resulted in an immediate increase in seizure behaviours which were associated with elevated EEG activity (Figure 6.3.1 and 2; no difference between groups). Diazepam administration, either alone or in combination with atenolol, resulted in an immediate drop in mean behavioural seizure score to 0.91 (pre-treatment score of 2.92). Atenolol significantly decreased seizure severity 170-180 minutes post-KA and reduced Level 4 behaviours compared to the saline group (Figure 6.3.1 and Table 6.3.1). Combination therapy significantly dropped behavioural score to an average of 0.8 at 60-180 minutes post-KA. This reduction in behavioural activity was associated with EEG activity attenuation back to baseline levels (Figure 6.3.2). Representative EEG traces and coinciding behavioural responses are demonstrated in Figure 6.3.3.

![Figure 6.3.1. Behavioural activity following intervention therapy (black line, with saline, diazepam, atenolol or diazepam+atenolol) at 60 minutes in KA (dashed line) seizure rats. *P<0.05 compared to baseline, *P<0.05 compared to saline-treated rats.]

<table>
<thead>
<tr>
<th>Table 6.3.1. Behavioural activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
</tr>
<tr>
<td>Cumulative</td>
</tr>
<tr>
<td>WDS</td>
</tr>
<tr>
<td>Level 4</td>
</tr>
<tr>
<td>Level 5</td>
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</table>

Behavioural score following intervention therapy (with saline, diazepam, atenolol or diazepam+atenolol) at 60 minutes in KA seizure rats over 120 min. The cumulative score was the sum of the maximum behaviour every minute. The total number of wet dog shakes, Level 4 and Level 5 behaviours recorded.
Figure 6.3.2. EEG activity following intervention therapy (with saline, diazepam, atenolol or diazepam+atenolol) at 60 minutes in KA-seizure (dashed line) rats. Histograms represent normalised power spectral density (PSD; mean ± SEM) for each 10 min bin across the delta, theta, alpha and beta frequency bands. # P<0.05 compared to baseline, + P<0.05 compared to saline-treated rats.
Figure 6.3. Representative EEG (grey trace) and behavioural activity (black dot plot) during seizures before and after intervention therapy in individual rats. Expanded waveforms demonstrate example EEG trace and corresponding seizure behaviour.
Figure 6.3.4. Heart rate (beats per minute, b.p.m.) following intervention therapy (black line, with saline, diazepam, atenolol or diazepam+atenolol) at 60 minutes in KA-seizure (dashed line) rats. *P<0.05 compared to baseline, *P<0.05 compared to saline-treated rats.

Figure 6.3.5. QTc interval following intervention therapy (black line, with saline, diazepam, atenolol or diazepam+atenolol) at 60 minutes in KA-seizure (dashed line) rats. *P<0.05 compared to baseline, *P<0.05 compared to saline-treated rats.

Figure 6.3.6. Normalised T wave amplitude following intervention therapy (black line, with saline, diazepam, atenolol or diazepam+atenolol) at 60 minutes in KA-seizure (dashed line) rats. *P<0.05 compared to baseline, *P<0.05 compared to saline-treated rats.
6.3.2 Cardiac function

As expected, KA seizures induced tachycardia in all animals during the initial 60 minutes (Figure 6.3.4). This was unabated by subsequent saline intervention. The reduction in seizure activity produced by diazepam intervention did not attenuate this tachycardic response, with a maximum HR of 483 ± 13 b.p.m. recorded. Atenolol treatment, alone or in combination, was effective at preventing tachycardia and dropped HR back to baseline levels (average of 363 ± 7 b.p.m.). QTc prolongation also occurred following KA administration (Figure 6.3.5). Diazepam administration failed to restore QTc interval which remained significantly prolonged until the 7 day (61 ± 1.7 ms) time point. Atenolol and diazepam+atenolol interventions were effective at bringing the QTc interval back to baseline levels for the remainder of the study. Continued T wave elevation was recorded in the saline and diazepam treatment groups (maximum increase of 51 or 84%, respectively; Figure 6.3.6). Seizures significantly elevated BP by 20-30% at the 60 minute recording point post seizure induction which was attenuated by atenolol administration (Figure 6.3.7). Diazepam administration resulted in further BP elevations to 156 ± 10 and 138 ± 6 mmHg at 3 and 24 hours, respectively. HRV analysis revealed that seizure activity resulted in a concurrent increase in sympathetic activity, as demonstrated by an increase in the LF/HF ratio by 1.5-2.6-fold at 60 minutes (Figure 6.3.8). Atenolol administration significantly inhibited the sympathetic effect, dropping LF (nu) by 45% and the LF/HF ratio to 0.42. Diazepam alone did not alter sympathovagal balance compared to saline treated animals.

Figure 6.3.7. Effect of seizure and intention therapy (with saline, diazepam, atenolol or diazepam+atenolol) on systolic blood pressure in rats. Presented as mean ± SEM, *P<0.05 compared to baseline, *P<0.05 compared to saline-treated rats.
Figure 6.3.8. The effect of seizure and intervention therapy on frequency analysis of heart rate variability. Data was analysed over a 5 min period throughout the study. Low frequency (LF; 0.04-0.5 Hz; A) and high frequency (HF; 0.5-3 Hz; B) data was presented as a normalised unit (nu) and LF/HF ratio (C). Data mean ± SEM. #P<0.05 compared to baseline.
Seizures were associated with an increase in plasma troponin I levels at 24 hours in the saline, diazepam and combination groups (3-4 fold) which was reduced in the atenolol treated group (NS compared to baseline; Figure 6.3.9). Echocardiography revealed dilation during diastole in the diazepam treated group by 39 % at 14 days ($P<0.05$ compared to atenolol, Figure 6.3.11). Diazepam animals also had significantly wider left ventricular posterior wall dimensions and elevated end volumes. Atenolol treatment alone reduced the extent of left ventricular dysfunction (Figure 6.3.10 and 11). Combination therapy was effective at preventing left ventricular dilation, reduced end volumes and preserved ejection fraction and fractional shortening.

![Graph showing plasma troponin I levels following seizures and therapeutic intervention with saline, diazepam, atenolol and diazepam+atenolol. *P<0.05 compared to baseline.](image-url)

**Figure 6.3.9.** Plasma troponin I (ng/ml) levels following seizures and therapeutic intervention with saline, diazepam, atenolol and diazepam+atenolol. *P<0.05* compared to baseline.
Figure 6.3.10. Left ventricular function following seizures and therapeutic intervention at 7 and 14 days. Dashed line represents control animal values presented in Chapter 5. Ejection fraction (EF) and fractional shortening (FS) were measured (mean ± SEM). *P<0.05 compared to saline treatment.

Figure 6.3.11 (Right). Left ventricular (LV) function following seizures and intervention at 7 and 14 days. Dashed line represents control animal systolic (s) and diastolic (d) values presented in Chapter 5. LV internal dimension (LVID), LV posterior wall thickness (LVPW), interventricular septum (IVS) and end volume (EV) were measured (mean ± SEM). *P<0.05 compared to saline treatment. +P<0.05 compared to diazepam treated
The effect of seizure activity on arrhythmia susceptibility was assessed by aconitine challenge. Seizures were associated with a reduced latency to arrhythmia onset and treatment with diazepam offered no protection (Figure 6.3.12). In conscious rats at 7 days, diazepam decreased latency to bigeminy by 36% compared to saline treated animals. Atenolol treatment was effective at increasing latency to arrhythmia onset at both time points. Combination therapy increased the latency to PVC and bigeminy by 51 and 49%, respectively, at 7 days compared to diazepam alone (Figure 6.3.12).

**Figure 6.3.12.** Shows the effect of aconitine on latency to arrhythmias (as demonstrated in Chapter 5) following seizures and intervention therapy. The 7 days rats were conscious and free-moving, while the 14 and 28 days rats were anaesthetised prior to aconitine administration. *P<0.05 compared to saline treated, +P<0.05 compared to diazepam treated.
6.3.3. Cardiac Immunohistochemistry and Histology

Histology at 7 and 14 days revealed significant evidence of fibrosis and oedema in the left ventricular myocardium (Figure 6.3.13). Hearts from saline treated animals showed evidence of apoptotic myocytes (0.5 ± 0.1 cells/mm²) and macrophage (73 ± 28 cells/mm²) infiltration at 14 days. Disappointingly, diazepam offered no cardiac protection against either fibrosis (6 ± 2%), oedema (19 ± 2%), apoptosis (0.5 ± 0.1 cells/mm²) or macrophage infiltration (58 ± 19 cells/mm²; Figure 6.3.13 and 14). Figure 6.3.15 is a representative cardiac micrograph from a 7 day diazepam treated animal with diffuse fibrotic deposition throughout the subendocardium. Atenolol, either alone or in combination with diazepam, was effective at preserving normal cardiac morphology (Figure 6.3.13 and 14). There was no significant difference in the extent of cardiac damage between 7 and 14 days in all treatment groups.

Figure 6.3.13. The effect of seizures of cardiac structure following intervention therapy. (A) Percentage of tissue positively stained for fibrosis. (B) Percentage of clear area used as a way of determining the presence of oedema. (C) The total number of apoptotic (ApopTag) and (D) macrophage (CD68) cells in the heart. *P<0.05 compared to saline.
Figure 6.3.14. Micrographs of the left ventricular subendocardium at 7 days following KA administration. Micro-lesions were found in the hearts of saline and diazepam treated rats. MSB staining for fibrosis and morphological structure (A-D); ApopTag positive cells (arrows; E-H) and macrophage infiltration (CD68 positive cells, I-L).
Figure 6.3.15. Example ventricular (A) micrograph from a diazepam seizure rat at 7 days with intersital (B) and perivascular (C) fibrosis.
6.3.4 Hippocampal Immunohistochemistry

There was evidence of immunolabelled apoptotic and microglial cells in the saline-treated rats which was significantly reduced by diazepam, atenolol and diazepam+atenolol therapies (Figure 6.3.16 and 17). Diazepam treatment significantly decreased apoptotic and CD11 positive cells by 4- and 10-fold, respectively, at 7 days in the ipsilateral hippocampus (Figure 6.3.16). Atenolol was also effective at decreasing apoptosis and CD11 by 2-7-fold and 3-6-fold, respectively. The same protective response was obtained following diazepam+atenolol combination treatment (Figure 6.3.16 and 17). Figure 6.3.18 demonstrates the different types of ApopTag staining observed in the dentate gyrus region of the hippocampus of seizure animals. ApopTag positive cells were concentrated to the pyramidal cell layer (Figure 6.3.18C) with occasional cells diffusely detected throughout the hippocampus (Figure 6.3.18D). CD11 positively stained cells were observed extensively within the hippocampus of seizure rats (Figure 6.3.19), with low levels detected in the treatment groups (Figure 6.3.19D).
Figure 6.3.16. Hippocampal micrographs staining for apoptosis in seizure animals following saline, diazepam, atenolol or diazepam+atenolol therapies.
Chapter 6: Therapeutic intervention during seizure-induced cardiomyopathy

Figure 6.3.17. Hippocampal micrographs staining for microglia (CD11) in seizure animals following saline, diazepam, atenolol or diazepam+atenolol therapies.
**Figure 6.3.18.** Micrographs of ApopTag positive cells within the hippocampus (dentate gyrus, ipsilateral side) of saline (A), diazepam (B), atenolol (C) and diazepam+atenolol (D) treated seizure rats at 7 days.
Figure 6.3.19. Micrographs of CD11 positive (microglia) cells within the hippocampus (dentate gyrus, ipsilateral side) of saline (A), diazepam (B), atenolol (C) and diazepam+atenolol (D) treated seizure rats at 7 days.
6.4. Discussion

This study investigates the use of intervention therapy in seizure-induced cardiomyopathy. Diazepam decreased seizure severity and hippocampal injury but surprisingly had no protective effect on cardiac function. Atenolol significantly reduced cardiac dysfunction and preserved normal cardiac morphology. Atenolol also reduced high level seizure behaviours and produced hippocampal protection. Combination therapy with atenolol on top of the diazepam proved effective at protecting both the heart and brain during seizure activity.

Diazepam therapy transiently reduced EEG activity and significantly decreased seizure behaviours, although animals appeared sedated and displayed compulsive eating (Wise & Dawson, 1974; Johnson, 1978). Previous literature studying diazepam effects, has confusingly reported either no change (Spracklen et al., 1970; Gerold et al., 1976; Clanachan & Marshall, 1980a; Clanachan & Marshall, 1980b; Marty et al., 1986; Sakamoto et al., 1990; Matzen et al., 1993; Kitajima et al., 2004; Conahan & Vogel, 1986; Taneyama et al., 1993), or decreases (Daniell, 1975; Bolme & Fuxe, 1977; Nakae et al., 1997) in HR in both animal and clinical studies (Table 6.4.1). It was surprising that seizure attenuation with diazepam failed to reduce the seizure-induced tachycardia in the present study. This finding in our rat model conflicted with older studies using diazepam, which report a reduction in HR by vagal mediated pathways and decreased plasma noradrenaline levels (Marty et al., 1986). Diazepam has also been associated with inhibition of L-type calcium channels (Nakae et al., 1997; Kanaya et al., 2002; Earl & Tietz, 2011) contributing to the negative inotropic and anti-arrhythmic effect (Spracklen et al., 1970; Daniell, 1975; DiMicco, 1987; Sakamoto et al., 1990; Kumagai et al., 1991; Matzen et al., 1993). Consequently, diazepam should have provided cardioprotection in the current study.

Further in-depth assessment of the literature, however, provides an explanation to the findings generated in this study. In addition to the actions already discussed above, diazepam has been shown to have phosphodiesterase (PDE) inhibitory effects, specifically the cardiac isoform PDE4 (Collado et al., 1998). This consequently produces a potentiation of the chronotropic and inotropic responses to noradrenaline and adrenaline on cardiac β-receptors (Marin & Hernandez, 2002). These direct cardiac effects of diazepam can escalate second messenger levels and may serve to override any beneficial effects obtained by ablating seizure activity (as demonstrated in Figure 6.4.1). Diazepam has also been reported to have vasolytic effect, by enhancing GABAergic inhibition of the vagal nerve (Hockman & Livingston, 1971; Keim & Sigg, 1973; DiMicco, 1987). This is supported by reports of HRV analysis conducted in patients. Administration of lorazepam or midazolam decreased HF power and increased LF/HF ratio,
indicative of sympathetic dominance (Agelink et al., 2002). The positive chronotropic effect of diazepam may be dose related. Gerold et al. (1976) reported that 0.3-1 mg/kg of diazepam had no influence on HR, however 10 mg/kg diazepam produced a rapid and sustained increase in HR in dogs. A similar trend was reported by Conahan and Vogel (1986) where diazepam (10 mg/kg) produced sustained tachycardia (Table 6.4.1).

Table 6.4.1. Summary of reported effects of diazepam administration on heart rate in animal and clinical studies.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Species</th>
<th>Dose</th>
<th>Conscious State</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increased Heart Rate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adinoff et al., 1992</td>
<td>Humans</td>
<td>~0.13 mg/kg iv</td>
<td>Conscious</td>
<td>↓ cardiac vagal tone</td>
</tr>
<tr>
<td>Agelink et al., 2002</td>
<td>Humans</td>
<td>~0.14 mg/kg iv</td>
<td>Anaesthetised</td>
<td>↓ vagal activity (HRV)</td>
</tr>
<tr>
<td>Hockman &amp; Livingston, 1971</td>
<td>Cats</td>
<td>0.18-3 mg/mg iv</td>
<td>Anaesthetised</td>
<td>Inhibited reflex vagal bradycardia</td>
</tr>
<tr>
<td>Kumagai et al., 1991</td>
<td>Humans</td>
<td>0.2 mg/kg iv</td>
<td>Conscious</td>
<td>No change in BP</td>
</tr>
<tr>
<td>Keim &amp; Sigg, 1973</td>
<td>Cats</td>
<td>0.3-3 mg/kg iv</td>
<td>Anaesthetised</td>
<td>Dose dependent ↑ in HR</td>
</tr>
<tr>
<td>Bataillard et al., 1990</td>
<td>Rats</td>
<td>0.3-3 mg/kg iv</td>
<td>Conscious</td>
<td>↑ ~2 hours. No change in BP</td>
</tr>
<tr>
<td>Conahan &amp; Vogel, 1986</td>
<td>Rats</td>
<td>1-10 mg/kg iv</td>
<td>Conscious</td>
<td>No change in BP</td>
</tr>
<tr>
<td>Mailliet et al., 2001</td>
<td>Rats</td>
<td>6 mg/kg ip</td>
<td>Conscious</td>
<td>↑ BP</td>
</tr>
<tr>
<td>Gerold et al., 1976</td>
<td>Dogs</td>
<td>10 mg/kg po</td>
<td>Conscious</td>
<td>↑ lasted 16 hours</td>
</tr>
<tr>
<td><strong>Decreased Heart Rate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yang et al., 1987</td>
<td>Rats</td>
<td>1-30 mg/kg</td>
<td>Conscious</td>
<td>Dose-dependent ↓ in BP &amp; HR</td>
</tr>
<tr>
<td>Daniell, 1975</td>
<td>Dog</td>
<td>2-20 mg/kg iv</td>
<td>Anaesthetised</td>
<td>↑ coronary blood flow and CO and ↓ BP</td>
</tr>
<tr>
<td><strong>No change in Heart Rate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kitajima et al., 2004</td>
<td>Humans</td>
<td>~0.08 mg/kg iv</td>
<td>Conscious</td>
<td>↓ BP and muscle sympathetic nerve activity. No change in HRV</td>
</tr>
<tr>
<td>Bolme &amp; Fuxe, 1977</td>
<td>Rats</td>
<td>0.1-1 mg/kg ip</td>
<td>Anaesthetised</td>
<td>Decreased BP</td>
</tr>
<tr>
<td>Matzen et al., 1993</td>
<td>Humans</td>
<td>0.15 mg/kg iv</td>
<td>Conscious</td>
<td>No change in HR or BP compared to controls</td>
</tr>
<tr>
<td>Gerold et al., 1976</td>
<td>Dogs</td>
<td>0.3-1 mg/kg po</td>
<td>Conscious</td>
<td>No change in BP</td>
</tr>
<tr>
<td>Taneyama et al., 1993</td>
<td>Dogs</td>
<td>0.4 mg/kg iv</td>
<td>Anaesthetised</td>
<td>No change in BP or renal nerve activity</td>
</tr>
<tr>
<td>Marty et al., 1986</td>
<td>Humans</td>
<td>0.4 mg/kg iv</td>
<td>Conscious</td>
<td>↓ in systolic BP</td>
</tr>
<tr>
<td>Sakamoto et al., 1990</td>
<td>Rabbits</td>
<td>0.5-1 mg/kg iv</td>
<td>Anaesthetised</td>
<td>↓ CO, increased TPR, No change in BP</td>
</tr>
<tr>
<td>Conahan &amp; Vogel, 1986</td>
<td>Rats</td>
<td>1-10 mg/kg iv</td>
<td>Anaesthetised</td>
<td>No change in BP</td>
</tr>
</tbody>
</table>

Diazepam administration is also associated with respiratory depression in animal (Lappierre et al., 2007) and clinical studies (Sykes & Okonofua, 1988; Leppik et al., 1983; Elterman, 1994; Appleton et al., 1995; Norris et al., 1999). In children receiving an acute rectal or iv dose of diazepam (max dose of 1 mg/kg) for convulsions, respiratory depression was recorded in 9% of cases. Clinically, diazepam administration results in respiratory depression in up to 15% of cases (Appleton et al., 1995). It is therefore plausible that the dose (5 mg/kg sc) of diazepam used in
the present rat model, may produce systemic anoxia provoking an increase in HR to maintain oxygen supply (Weiskopf et al., 2003 and 2002)

In addition to producing tachycardia, the present study also found that diazepam potentiated the seizure-induced hypertension in the conscious rat. This was surprising as previous literature (Table 6.4.1) has indicated that diazepam produces a hypotensive effect (Sigg et al., 1971; Daniell, 1975; Bolme & Fuxe, 1977; Marty et al., 1986; Sunzel et al., 1988; Kitajima et al., 2004) or no pressor change (Conahan & Vogel, 1986; Kumagai et al., 1991; Taneyama et al., 1993). It is possible that the hypertensive response seen following diazepam is mediated at the level of the nucleus of the solitary tract (NTS). The NTS is involved in autonomic regulation of cardiovascular function and allosteric stimulation of GABAergic activity results in disinhibition of sympathetic outflow, increasing arterial pressure (Barron et al., 1997).

Figure 6.4.1. Proposed effect of diazepam on the function of cardiac cells. AC: adenlyate cyclase; ACh: acetylcholine, GIRK: protein-coupled inwardly-rectifying potassium channels, NA: noradrenaline, PKA: Protein Kinase A, SR: sarcoplasmic reticulum.
Intervention therapy with atenolol was effective at preventing cardiac dysfunction during seizures in this study. Atenolol treatment reduced tachycardia and hypertension which preserved left ventricular function and cardiac morphology. Atenolol administration also prevented fibrotic deposition in conjunction with decreased susceptibility to aconitine-induced arrhythmias at 7 and 14 days. This supports clinical data where atenolol reduces the incidence, frequency and severity of ventricular arrhythmias by increasing the ventricular fibrillation threshold (Vincent et al., 1983; Rossi et al., 1983). By preserving cardiac function, atenolol potentially have reduced cerebral hypoxia, thereby decreasing seizure-induced hippocampal injury. These findings strongly suggest that atenolol has therapeutic benefit and should be considered for use in combination with current anti-epileptic medications. As demonstrated in the present study, combination therapy with diazepam and atenolol proved effective at protecting both the heart and brain during sustained seizure activity. Chugh et al. (1991) reported a similar effect where diazepam administration was effective at reducing aminophylline-induced seizures in mice but offered no protection against mortality. However when diazepam (10 mg/kg) was administered in combination with atenolol (5 mg/kg) it produced complete protection against convulsions and death (Chugh et al., 1991). In audiogenic mice, propranolol (30–50 mg/kg, ip) and metoprolol (50 mg/kg, ip) also significantly reduced seizure severity and served to potentiate the anti-epileptic effects of diazepam when used as an adjunct therapy (De Sarro et al., 2002). Disappointingly, substitution of these β-blockers with atenolol (either alone or in combination with diazepam; 1–50 mg/kg, ip) failed to offer the same benefit over the short 60 minute recording period (De Sarro et al., 2002). A similar effect was reported in an electroshock convulsion model in mice, propranolol (5 mg/kg, ip) and metoprolol (50 mg/kg, ip) increased the anticonvulsant effect of diazepam, although atenolol (10 mg/kg, ip) produced no further protection (Luchowska et al., 2002).

This chapter clearly demonstrates the importance of protecting both the heart and brain during seizures. Atenolol was effective at preserving normal cardiac function and appeared to potentiate the anticonvulsant effect of diazepam. Importantly, atenolol administration with diazepam, prevented the cardiac dysfunction seen in the diazepam treated animals. Therefore, atenolol should be considered clinically in epileptic patients, to reduce the cardiac dysfunction reported as a consequence of seizures and antiepileptic therapy.
Chapter 7

Final Discussion and Conclusions
7.1. Summary of results

The results in this study clearly demonstrate that seizures are associated with cardiac dysfunction, particularly changes in heart rate, QTc interval and blood pressure. Seizure-induced cardiac dysfunction resulted in significant structural damage as early as 48 hours, which was still present up to 28 days after the original seizure induction. These various studies reported in the experimental chapters demonstrate that altered autonomic function is involved in the pathology of seizure-induced cardiomyopathy. The seizure-induced tachycardia which ensued resulted in the development of dilated cardiomyopathy with significant cardiac structural injury. The development of micro-lesions and fibrotic deposition is suggested to contribute to left ventricular dysfunction and an increased susceptibility to arrhythmia induction. This thesis has consistently demonstrated that atenolol administration (prophylactic or intervention) offers significant protection against seizure-induced cardiomyopathy. Atenolol treatment during seizures also reduced EEG and behavioural score severity and protected the hippocampus. Atenolol, therefore, should be considered for clinical use, prophylactically in epilepsy or as a rescue intervention during SE. Importantly, this study clearly demonstrated that atenolol in combination with diazepam offers superior therapeutic benefit, over either monotherapy.

7.2. Comparison of kainic seizure models

Systemic administration of KA resulted in a behavioural hypoactive period associated with an immediate 30% drop in HR. This bradycardia coincided with an increase in the theta frequency band and the development of bradyarrhythmias. Conversely, direct administration of KA into the hippocampus, resulted in an immediate increase in behavioural activity and heart rate, with no evidence of bradycardia (Appendix 8.4, Figure 8.4.1 and 2). However, there was no significant difference in the extent of structural damage between the two different KA administration routes (Table 8.4.1). A possible explanation for these differences is that systemic administration of KA produces peripheral effects. Early evidence of peripheral glutamatergic receptor subunits was found in ganglia cells, conducting fibres, nerve bundles and cardiomyocytes in the hearts of rats (Gill et al., 1998) although studies by our group failed to find any evidence of a direct functional effect on isolated cardiac haemodynamics, using KA (50 μM) or domoic acid (1-5 μM; Vranyac-Tramoundanas, 2007; Vranyac-Tramoundanas et al., 2011). Experimental evidence (autographic and mRNA) has also demonstrated the presence of ionotropic glutamate receptors on the vagus nerve (Lewis et al., 1987; Agrawal & Evans, 1986; Cincotta et al., 1989; Shigemoto et al., 1992; Sato et al., 1993; Carlton, 2001). Specifically,
AMPAs and kainate receptors have been located in the dorsal root ganglion, spinal cord, nodose ganglion and on the peripheral vagus nerve trunks (Petralia & Wenthold, 1992; Furuyama et al., 1993; Sivaro et al., 1999; Carlton, 2001; Slattery et al., 2006). Evidence to negate the suggestion that systemic KA has a direct cardiac effect and support the concept of a vagal mediated response to KA is provided in the ipratropium study in Chapter 3 (Figure 3.3.6C). Ipratropium is a hydrophilic non-selective muscarinic antagonist indicated to have no CNS effects and was consequently used in this study to inhibit the effects of cardiac vagal activity. By preventing the extent of bradycardia in response to systemic KA administration, ipratropium antagonism of muscarinic receptors confirmed that the bradycardic response was mainly due to enhanced vagal activity and not reduced sympathetic activity. This finding was supported by HRV analysis, where parasympathetic dominance was recorded. Pre-treatment with diazepam (5 mg/kg, sc; Appendix 8.2) prior to systemic KA administration was effective at preventing seizure activity (behavioural and EEG activity; Figure 8.2.1) but did not alter KA-induced HR changes. This finding again supports the concept that KA is acting directly on the vagus nerves to produce bradycardia, rather than seizure-induced bradycardia.

There is an absence of data in the literature examining elimination of KA following systemic administration. However, studies with domoic acid (up to 1 mg/kg), a structural analogue of KA, indicate that this excitotoxin undergoes rapid elimination in rats, with 95% of the compound excreted within 2 hours (Truelove & Iverson, 1994; Suzuki & Hierlihy, 1993; Maucher & Ramsdell, 2005). Studies in rats have reported that domoic acid has a half-life of approximately 20 minutes and is excreted unchanged (Suzuki & Hierlihy, 1993; Truelove & Iverson, 1994; Iverson & Truelove, 1994). This suggests that if the pharmacokinetics of KA are similar to those reported for domoic acid, then it is plausible that the bradycardia may be induced by KA acting directly on the vagus nerve. Furthermore, any ensuing changes in HR may therefore be a consequence of the residual seizure activity resulting from KA induced hyperactivation of the CNS. This theory is supported by EEG spiking and high level seizure behaviours developing after the bradycardia, coinciding with the tachycardia over the 60-180 minute period post-KA.
Table 7.1: Summary of reported glutamatergic modulation of cardiac autonomic function.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Dose</th>
<th>HR</th>
<th>BP</th>
<th>Further comments and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sympathetic Responses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KA</td>
<td>2 nmol</td>
<td>↑</td>
<td>↑</td>
<td>↑ RSNA; Zhong et al., 2008; Jin &amp; Rockhold, 1989</td>
</tr>
<tr>
<td>NMDA</td>
<td>50-100 pmol</td>
<td>-</td>
<td>-</td>
<td>Goren et al., 2000b</td>
</tr>
<tr>
<td>NMDA</td>
<td>1-10 nmol</td>
<td>↑</td>
<td>↑</td>
<td>↑ splanchnic nerve activity; Kawabe et al., 2008.</td>
</tr>
<tr>
<td>Glutamate</td>
<td>15-50 nmol</td>
<td>↑</td>
<td>↑</td>
<td>↑ noradrenaline; Martin &amp; Haywood, 1992</td>
</tr>
<tr>
<td><strong>Dorsomedial hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KA</td>
<td>0.1-1.0 pmol</td>
<td>↓</td>
<td>↓</td>
<td>Soltis &amp; DiMicco, 1992a; Rockhold et al., 1987</td>
</tr>
<tr>
<td>KA</td>
<td>0.13 nmol</td>
<td>↑</td>
<td>↑</td>
<td>Rockhold et al., 1987</td>
</tr>
<tr>
<td>KA</td>
<td>0.4 nmol</td>
<td>-</td>
<td>-</td>
<td>Rockhold et al., 1987</td>
</tr>
<tr>
<td>NMDA</td>
<td>1-100 pmol</td>
<td>↑</td>
<td>-</td>
<td>Goren et al., 2000a; Soltis &amp; DiMicco, 1992a</td>
</tr>
<tr>
<td>AMPA</td>
<td>0.3-3 pmol</td>
<td>-</td>
<td>-</td>
<td>Soltis &amp; DiMicco, 1992a</td>
</tr>
<tr>
<td><strong>Intermedial lateral column</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>200 pmol</td>
<td>↑</td>
<td>-</td>
<td>Arnolda et al., 1996</td>
</tr>
<tr>
<td>KA</td>
<td>200 pmol</td>
<td>↑</td>
<td>-</td>
<td>Arnolda et al., 1996</td>
</tr>
<tr>
<td>KA</td>
<td>1-50 pmol</td>
<td>-</td>
<td>-</td>
<td>da Silva et al., 2006</td>
</tr>
<tr>
<td>KA</td>
<td>0.03-0.3 nmol</td>
<td>↑</td>
<td>-</td>
<td>Goodwin &amp; Barr, 1998</td>
</tr>
<tr>
<td>NMDA</td>
<td>3-12 pmol</td>
<td>↑</td>
<td>↑</td>
<td>da Silva et al., 2006</td>
</tr>
<tr>
<td><strong>Amygdala</strong></td>
<td>Glutamate</td>
<td>2.5 μmol</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td><strong>Periaqueductal gray</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>10-500 pmol</td>
<td>↑</td>
<td>↑</td>
<td>Chamberlin &amp; Saper, 1992</td>
</tr>
<tr>
<td><strong>Striatum (CaP)</strong></td>
<td>KA</td>
<td>1 pmol</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td><strong>Ventral medulla</strong></td>
<td>KA</td>
<td>40 nmol</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td><strong>Lateral septum</strong></td>
<td>KA</td>
<td>1-5 nmol</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td><strong>Prefrontal cortex</strong></td>
<td>Glutamate</td>
<td>3-150 nmol</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td><strong>Parasympathetic Responses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nucleus Ambigus</strong></td>
<td>KA</td>
<td>500 ng</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>NMDA</td>
<td>40-80 pmol</td>
<td>↓</td>
<td>↓</td>
<td>Yan et al., 2009</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-10 pmol</td>
<td>↓</td>
<td>↓</td>
<td>Yan et al., 2009</td>
</tr>
<tr>
<td><strong>Nucleus of the solitary tract</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KA</td>
<td>8-17 pmol</td>
<td>↓</td>
<td>↓</td>
<td>Kubo &amp; Kihara, 1991</td>
</tr>
<tr>
<td>KA</td>
<td>2 nmol</td>
<td>-</td>
<td>↓</td>
<td>↓ Sympathetic nerve activity; Duan et al., 2009</td>
</tr>
<tr>
<td>NMDA</td>
<td>-300 pmol</td>
<td>↓</td>
<td>↓</td>
<td>Reis, 1981; Le et al., 1989</td>
</tr>
</tbody>
</table>

Agonists administered directly into the specific brain region. CuP: Caudate putamen complex (Talman et al., 1981; Paton et al., 1990; Soltis & DiMicco, 1992b; Soltis, 1992; Vardhan et al., 1993; Shields, 1993b; Devinsky, 2004).

Intrahippocampal administration of KA on the other hand, produced an immediate tachycardic and hypertensive response. This is most likely a consequence of glutamatergic activation of sympathetic nuclei surrounding the hippocampus (Figure 7.1). Studies examining direct administration of glutamate agonists into these various neuronal regions are summarised in Table 7.1 and Figure 7.1. These studies demonstrate that glutamatergic activation of nuclei in close proximity to the hippocampus, mediate increases in HR and BP while the rhombencephalon (hindbrain) regions are associated with parasympathetic activity (Figure 7.1 and Table 7.1). This distribution may also be involved in how systemic KA produced bradycardia which was not seen following intrahippocampal KA infusion.
Figure 7.1. Illustration of ionotropic glutamatergic receptor activation of specific regions in the rat brain. Demonstrates the effect of glutamate agonist administration on sympathetic (red) and parasympathetic (green) activity Prefro. Cortex: Prefrontal cortex; CPu: Caudate putamen of the striatum; L Sep: Lateral septum; PVN: Paraventricular nucleus; Amy: Amygdala; DMH: dorsal medial hypothalamus; PAG: Periaqueductal gray; PBN: Parabrachial nucleus; NTS: nucleus of the solitary tract; 10N: vagal nerve; NAm: Nucleus ambiguus; RVLM: rostral ventrolateral medulla; DVN: dorsal vagal nucleus. CVLM: caudal ventrolateral medulla. Data from Table 7.1; rat brain image adapted from (Paxinos & Watson, 2007).

7.3. Adrenergic modulation in Seizure

This thesis has clearly demonstrated the benefit of modulating adrenergic activity with clonidine and atenolol during seizure. Adrenergic receptors are distributed throughout the hippocampus (Figure 7.2). Alpha 2 adrenoceptors are moderately dispersed throughout the CA1 and CA3 regions and are expressed pre- and post-synaptically. Clonidine administration produced an overall reduction in seizure severity, most likely as a consequence of both decreased pre-synaptic noradrenaline release and reduced post-synaptic cAMP levels (Figure 7.3). Beta 1 adrenoceptors are expressed in high levels in the CA3 hippocampal region with moderate levels in the dentate gyrus (Figure 7.2).
Noradrenaline has a variety of effects in the hippocampus, with the majority of actions involving enhanced excitability of pyramidal cells (Raman et al., 1996; Katsuki et al., 1997; Hu et al., 2007; Tenorio et al., 2010). Noradrenergic supply to the hippocampus is almost exclusively derived from the locus ceruleus (Fillenz, 1990). Microinjections of glutamate (0.5 mM) into the locus ceruleus of anaesthetised rats, produced CA1 excitability (Olpe et al., 1986). Hippocampal β-adrenergic stimulation has been reported to be involved in NMDA receptor modulation, via cAMP-dependent protein kinase activation, whilst noradrenaline administration has been found to increase phosphorylation of AMPA receptors (Dunwiddie et al., 1992; Raman et al., 1996; Hu et al., 2007). In mice, Hu et al. (2007) demonstrated that noradrenaline administration or stress (induced by fox urine) resulted in phosphorylation of the GluR1 subunit at Ser845 and Ser831. These authors also reported that direct noradrenaline administration facilitated long term potentiation in hippocampal slices (Hu et al., 2007). These results were supported by Tenorio et al. (2010), who further suggested that β-adrenoceptor modulation facilitates long term potentiation by increasing trafficking of AMPA receptors to the cell surface. The literature
suggests that phosphorylation of NMDA receptor modulation is dependent on which NMDA receptor subunits are present (Grant et al., 1998; Salter et al., 2009). α1-adrenoceptor mediated activation of protein kinase C (PKC) can result in phosphorylation of NMDA receptors. PKC produces enhanced NMDA currents at NR2A and NR2B subunits, which is not seen in receptors containing the NR2C or NR2D subunits (Salter et al., 2009). Phosphorylation of the NR2C subunit produces internalisation of the NMDA receptor while NR2B-phosphorylation has been reported to result in NMDA receptor stabilisation with the cell membrane (Chen and Roche, 2007). These studies suggest that enhanced noradrenaline release during a seizure, may be implicated in enhanced neuronal excitability via increased AMPA and NMDA receptor activation. Therefore, hippocampal β1 (and β2) blockade, may exert anti-convulsant effects via reduced phosphorylation of AMPA and NMDA receptors, thereby decreasing Na+ and Ca2+ currents (Figure 7.3; Chen & Roche, 2007; Hu et al., 2007; Salter et al., 2009). As KA produces excitotoxicity of the hippocampal CA1, CA3 and dentate gyrus (Wang et al., 2005) decreased adrenergic stimulation of these regions using sympatholytics, is postulated to mediate seizure attenuation in a number of the experimental studies conducted in this thesis.

Enhanced intracellular Ca2+ levels are implicated in excitotoxic mediated cell death (as described in Section 1.4.2). Initiation of apoptosis, through the activation of proteases and protein kinases, is facilitated by mitochondrial Ca2+ overload producing a release of reactive oxygen species and caspase cofactors, such as cytochrome c (Brooke et al., 2004; Campanella et al., 2004). Additionally, elevated intracellular Ca2+ levels also mediate inflammation by activation of calcineurin and IκB kinase, producing transcription of inflammatory mediators, including interleukins and tumour necrosis factor-α (Feske et al., 2007 and 2013; Sperlagh and Csolle; 2011). Based on this knowledge it is therefore hypothesised that atenolol may decrease seizure-induced apoptosis and inflammatory responses by preventing β-adrenergic mediated phosphorylation of AMPA and NMDA receptors (Figure 7.3).
Chapter 7: Final Discussion and Conclusion

**Figure 7.3.** Diagrammatic representation of the proposed mechanism of synaptic adrenergic modulation of glutamatergic receptors. KA: Kainate receptor; Glut: glutamate; NA: noradrenaline; P: phosphate; PKA: protein kinase A; PKC: protein kinase C; VGCC: voltage gated calcium channels. (MacDonald et al., 1989; Chen & Roche, 2007; Hu et al., 2007; Salter et al., 2009).

Pilot studies examining $^{14}$C-sucrose distribution following intravenous administration showed an increase in blood brain barrier permeability within 3 hours of intrahippocampal KA administration (Appendix 8.5, Figure 8.5.1). This suggests that the drop in seizure behaviours and EEG at 2.5-3 hours post-KA may be a direct consequence of atenolol permeation through a compromised blood brain barrier into the hippocampus. Conversely, no significant increases in $^{14}$C-sucrose loading into the brain occurred, following subcutaneous KA administration, suggesting that the atenolol-mediated drop in seizure severity may be due to other mechanisms. Chapter 3 has also suggested that a relationship exists between heart rate and seizure

**Figure 7.4.** Relationship between seizures, heart rate and neuronal damage.
severity, which was also supported in the subsequent intervention study in Chapter 6. Appendix 8.1.3 demonstrated a correlation $R^2=0.56$ between HR and behavioural scores in the intervention study. Correlations were also seen between hippocampal apoptosis and both mean seizure score and elevated HR (Figure 8.1.10). These findings suggest the existence of a feedback pathology involved in seizure-induced cardiomyopathy (Figure 7.4). As discussed, seizures directly cause hippocampal damage through excitotoxic mechanisms. The resulting injury can cause propagation of seizure activity to other brain regions through cell injury-mediated release of Ca$^{2+}$ and glutamate signalling transmitters (Brorson et al., 1997; Lerma, 2009). Consequently, sustained seizure severity may directly increase HR through activation of autonomic nuclei and catecholamine release (Jin & Rockhold, 1989; Soltis & DiMicco, 1992; Martin & Haywood, 1992; Zhong et al., 2008; Kawabe et al., 2008; Goren et al., 2000b). This elevation in HR can lead to reduced cerebral flow and hypoxia which causes further neuronal hyperactivation and injury (Downing et al., 1963; Low et al., 1999).

7.4. Adrenergic mediated cardiac dysfunction. The cardiac pathology observed in this study, consequent to seizure, is similar to post-mortem reports from epileptic patients (Kloster et al., 1999; Natelson et al., 1998; Stollerberger & Finsterer, 2004). This pathology included oedema, inflammatory cell infiltration, myocyte vacuolisation and perivascular and interstitial fibrosis. Previous chapters have discussed, how this cardiac injury may occur as a consequence of seizure-induced tachycardia. This is supported by clinical and animal studies, where sustained tachycardia produces cardiac structural damage, increased susceptibility to arrhythmias and left ventricular dilation (Morady et al., 1985; Khanis et al., 2005; Metcalf et al., 2009a). It is proposed that this occurs due to increased cardiac oxygen demand associated with decreased coronary blood and potentiated by catecholamine-induced vasospasm (Lyon et al., 2008). This can result in the presence of micro-infarcts and micro-lesions as demonstrated by myocyte vacuolisation, necrotic cell death and inflammation (Kajstura et al., 1996).

Another possible mechanism for seizure-induced cardiac damage, is direct β-adrenergic stimulation. Short-term activation of β-adrenoceptors enhances cardiac function, while long term stimulation mediates β-adrenergic-induced cell death (Whelan et al., 2013). In the intrahippocampal kainic acid model, noradrenaline levels were significantly increased at 3-48 hours post seizure induction, which may result in myocyte dysfunction and death via enhanced β-adrenergic stimulation. Hyperadrenergic states can induce cardiomyocyte cell death through oxidative stress producing reactive oxygen species and energy (ATP) loss. These mechanisms
can result in mitochondrial Ca\(^{2+}\) overloading, initiating necrosis and apoptosis (similar to the mechanism described above consequent to excitotoxicity, Goldspink, 2004; Whelan et al., 2013).

The devolvement of necrosis is also associated with inflammatory cell infiltration, troponin I release and fibrotic deposition (Neri et al., 2007; Zhang et al., 2008).

In the present study, there was limited ApopTag positive cells (0.5 ± 0.1 cells/mm\(^2\)) detected throughout the myocardium, this may be due to the late time frame. Kajstura et al. (1996) found that cardiac apoptosis peaked within 5 hours of a myocardial infarction in rats and had dissipated by 24 hours. These results were supported by Goldspink et al. (2004), where a single injection of isoprenaline (5 mg/kg, sc) produced an increase within an hour (peaking at 3-6 hours). Noradrenaline administration (3 mg/kg, ip) in rats, resulted in necrosis and hypercontraction bands within 1 hour and by 8 hours there was evidence of macrophage infiltration (Neri et al., 2007). These results suggest that β-adrenergic hyperstimulation can directly produce cell death and inflammation.

### 7.5. Merits and limitations of non-invasive measures of autonomic function

Heart rate variability analysis has been advocated in the literature as a non-invasive measure of autonomic function (Acharya et al., 2006). HRV was therefore utilised in this study in order to provide multiple temporal measures of cardiac autonomic function over the duration of the seizure response. This technique has provided significant insight into cardiac autonomic modulation during seizures. HRV analysis demonstrated that systemic administration of KA produced increased autonomic function of both systems with parasympathetic dominance. This finding further supports the theory that the bradycardia recorded following systemic KA, was a consequence of increased vagal activity and not decreased sympathetic modulation. In comparison, intrahippocampal administration of KA produced increased sympathetic activity at 60 minutes, 48 hours and 7 days following seizure induction. This conclusion from the HRV study was confirmed by the data obtained in the autonomic blockade experiments with ipratropium and atenolol. An advantage of pharmacological blockade with ipratropium and atenolol is that it allows for assessment of each branch of the autonomic system. Seizure activity produced a drop in parasympathetic responsiveness to ipratropium blockade and an increased responsiveness to atenolol blockade at 48 hours (Figure 5.3.8A). A major benefit of pharmacological blockade of the autonomic (vagal sympathetic) innervation to the heart is that this procedure allows estimation of intrinsic HR. A reduction in the intrinsic HR is associated with aging, myocardial ischaemic damage and diabetes (Marcus et al., 1990). In this seizure model, intrinsic HR decreased at the 48 hour and 7 days recording, suggesting nodal pace maker
injury. The continued presentation of normal basal HR with decreased intrinsic HR, suggests that there is a shift in control of autonomic innervation in favour of sympathetic dominance. This finding is concurrent with the evidence gained from the HRV study. The advantages and disadvantages of HRV and vagal sympathetic effect are summarised in Figure 7.5. The use of both methods in the present study, served to provide insight into how seizure activity produces acute and chronic changes in autonomic function.

**ADVANTAGES**

**Heart Rate Variability**
- Does not require pharmacological modulation
- Multiple sampling can be taken to assess when subjects are most susceptible to autonomic dysfunction
- Impacted by physiological factors which alter HR
  - Eg respiration and baroreflex
- More accurate in a clinical setting
  - Patient is seated (no movement or postural changes)
  - Respiration can be controlled

**Pharmacological denervation**
- Non-invasive
- Can be used in conscious subjects
- Able to individually assess parasympathetic or sympathetic activity
- Accurate for assessing cardiac autonomic function
  - Only measuring the change in HR
- Allows the intrinsic HR to be assessed

**DISADVANTAGES**

**Heart Rate Variability**
- Does not give an indication of sympathetic modulation
  - LF band also impacted by baroreflex and RAAS modulation
- Relies on multiple assumptions in terms of where physiological responses fall within the frequency band
  - Does not account for individual variation
- Can be inaccurate if there are missing heart beats
  - However short term recordings can reduce this
- Frequency band analysis affected by age, sex species and circadian rhythm

**Pharmacological denervation**
- Accuracy requires establishment of a baseline (ie pre-seizure) recording
- Requires pharmacological modulation
- Can not be performed multiple times throughout the day
  - Requires a washout period
- Multiple assessment of VSE can alter the progression of the cardiac disease
  - Esp if studying hyperactivation of the sympathetic system

*Figure 7.5. Advantages and disadvantages of heart rate variability analysis compared to pharmacological denervation with ipratropium and atenolol. RAAS: renin angiotensin aldosterone system.*
7.6. Improvements and Future Directions

7.5.1. Model of seizure activity
Intrahippocampal administration of KA, produced a reliable model of seizure-induced tachycardia. Intrahippocampal administration of KA has the additional benefit of ensuring that the small amount of KA is not producing systemic effects. Future experiments could continue with this model to assess acute seizure-induced cardiomyopathy or intrahippocampal KA administration could be used to produce a kindling seizure model which represents a better model of chronic epilepsy. A disadvantage of kindling models is that they have a high mortality rate, although this could be used to our advantage to assess the risk of seizure-induced sudden cardiac death.

A limitation of this study, is that the long term effect (14 and 28 days) of seizures on ECG activity was not assessed. The reason for this is that secure placement of the cardiac electrodes (to reduce movement artefacts during seizures) means that it cannot accommodate for the rapid growth in this age group of rats. Therefore to assess susceptibility to arrhythmias the rats were anaesthetised which increased the latency to arrhythmia onset in the control animals. Future models should consider other methods of ECG recording such as non-invasive ECG recording chambers (such as ECGenie by Mouse Specifics).

7.6.2. Further investigation into seizure-induced dysfunction
A major benefit of the present study is that it allows cardiac function to be assessed in a conscious model of seizure. However, in order to achieve better insight into the direct role of seizure-induced cardiac autonomic dysfunction, the rats could be anaesthetised and cardiac sympathetic and vagal nerve activity investigated. This study used pharmacological blockade of autonomic activity to determine the intrinsic heart rate, however this method could be substituted using a Langendorff preparation. Cardiac $^{123}$I-metaiodobenzylguanidine (MIBG) is taken up by myocardial sympathetic nerve terminals (Minardo et al., 1988; da Silva et al., 2013) and could be used in the present study as a means of measuring seizure-induced sympathetic alterations.

A limitation of this study is the use of domitor to sedate the rats for echocardiographic recordings. Domitor could be substituted for isoflurane (1.5-2.5%; Riha et al., 2012) or ketamine and midazolam (Sabatini et al., 2013), which are associated with less cardiovascular side effects, allowing for multiple recordings to be taken within a rat. Although midazolam may produce anticonvulsant effects and alter progression of the disease.
Future studies should investigate oxygen saturation, to determine the involvement of respiratory dysfunction during seizure. In the present study, a tail clip was used to assess oxygen saturation, however the results proved unreliable (data not shown). The use of blood sampling or telemetric implantation could be superior alternatives.

Furthermore investigations into how seizures affect the baroreflex response may be of interest. Baroreflex sensitivity can be examined pharmacologically with phenylephrine (an $\alpha_1$ adrenergic agonist, vasoconstrictor) and nitroprusside (a nitric oxide donor, vasodilator).

A potential limitation of this study is the use of an ApopTag kit. The terminal transferase-mediated nick end labelling technique (TUNEL) stain has been scrutinised for its accuracy at detecting apoptotic cells. This stain targets DNA fragmentation which can also occur as a consequence of necrosis or replication and repair (Labat-Moleur et al., 1998). The ApopTag kit has been reported to have good reproducibility but does show affinity for necrotic cells (Garrity et al., 2003). TUNEL staining, if optimised properly, is a good option for visualising the presence of cell death. However, in order to confidently distinguish between apoptosis and necrosis, Western blotting should be undertaken to assess caspase levels in both hippocampal and cardiac tissue. Furthermore, a limitation of this study, is that I have not co-stained for neuronal cells in the hippocampus. The morphology of the cells, suggests that pyramidal cells and interneurons are apoptotic post-KA administration, however co-staining with Anti-NeuN, would ensure that the apoptotic cells are not a consequence of apoptotic microglia. Receptor interaction protein (RIP) kinase can be used as a marker of necrosis via protein densitometry, however this would not provide us with information of where these necrotic areas are occurring within the myocardium.

To investigate the molecular mechanism of seizure-induced cardiac dysfunction, $\beta$ adrenergic, L-type calcium channels and Kv potassium receptor levels should be examined by protein densitometry. Decreased expression of Kv4.2 receptors have been reported following pilocarpine-induced SE and have been associated with increased arrhythmia risk (Bealer et al., 2010). This is supported by Glasscock et al., (2010) where Kv1.1 potassium channel deficiency produces seizures and interictal AV blocks, which may be implicated in SUDEP. It would be interesting to note, whether enhanced sympathetic activity leads to a modulation in expression of these channels.

7.6.3. Therapeutic intervention in seizure-induced cardiomyopathy

In the current study, the dose of diazepam chosen to attenuate seizure activity, successfully prevented typical seizure behaviours such as wet dog shakes and myoclonic jerks. However, it
was noted diazepam administration produced an increase in behaviours such as abnormal resting, mastication and a loss of balance, which explains why seizure behaviours had not returned to baseline. It would therefore be interesting to determine if a lower dose of diazepam would produce the same neuronal protection without the associated side effects, including elevated HR.

In this study, atenolol treatment decreased seizure severity. The use of $^{14}$C-sucrose to assess blood brain barrier (BBB) permeability, suggested that intrahippocampal KA decreased the integrity of the BBB. High-performance liquid chromatography could be used in the future, to determine whether atenolol is in the brain where it may be directly inhibiting β receptors.

Future experiments in our laboratory will investigate whether atenolol in combination with conventional antiepileptics will produce the same benefit observed with diazepam in the present study. Valproate and carbamazepine are the most commonly prescribed anti-epileptic agents. Therefore it would be clinically relevant to determine whether atenolol adjunct therapy will offer superior protection against seizure-induced cardiomyopathy. Previous studies by Luchowska et al. (2001, 2002) demonstrated that β blockade, with metoprolol and propranolol, enhanced the anticonvulsant effect of carbamazepine, phenytoin and MK801 (an NMDA receptor antagonist) suggesting that β-blockers are a clinically viable option in epilepsy.

Experiments in our laboratory will also investigate the use of other beta blockers, either alone or in combination with conventional antiepileptics to investigate the anticonvulsant effect of β-receptor blockade. Carvedilol is a non-selective beta blocker with α1 antagonist properties, used to treat arrhythmias, hypertension and ischaemic heart disease. Low dose administration of carvedilol will be utilised in future studies to attenuate increases in heart rate and blood pressure associated with seizure, and reduce the extent of ischaemic damage. Carvedilol is a lipophilic compound which is hypothesised to produce greater anticonvulsant properties than atenolol. Combination of carbamazepine with β-blockers should be trialled to reduce cardiac morbidity in epilepsy.

While, it is paramount that sympatholytic intervention remains contraindicated in patients at risk of bradycardia and bradyarrhythmia, this data strongly suggests that atenolol can add cardioprotective value to current antiepileptic treatment strategies conducted in patients suffering frequent seizures. Intervention trials with atenolol in status epilepticus should include serial assessments of cardiac function using ECG, echocardiography and injury markers (such as troponin I) in order to provide much needed information on the pathology and validation of this pharmacological approach. Furthermore, prophylactic treatment with atenolol may also have a
role in epilepsy patients presenting with a number of risk factors for sudden unexpected death (SUDEP; Surges et al., 2009).

7.6.4. Investigation into mechanism behind systemic kainic acid

Future studies should examine the effect of subcutaneous systemic KA on vagal nerve activity. These investigations will provide clinically relevant information relating to reports of accidental exposure to excitotoxins such as the kainic acid and domoic acid incidences (Iverson et al., 1989; Iverson & Truelove, 1994; Ross et al., 2000; Silvagni et al., 2005). Experiments in this area could examine the effect of a vagotomy in a rat prior to KA administration to determine if vagal denervation will attenuate the bradycardic effects systemic KA.

7.7. Final conclusion

These experiments clearly demonstrate that KA-induced seizures results in a significant alteration in autonomic function, contributing to the development of tachycardia and myocardial injury. Systemic KA administration produces a reliable and consistent model of seizure activity. Animals consistently displayed the same progression of seizure behaviours, however this study suggests systemic KA protocols may produce limitations as a model of seizure-induced cardiomyopathy. Intrahippocampal KA is an improved reliable model for examining the peripheral consequences of seizure. This model is particularly valuable for studying seizure-induced cardiac dysfunction, as it replicates the present cardinal clinical features of tachycardia, arrhythmia and cardiac structural injury, whilst avoiding the systemic problems associated with chemical-induced seizure models. Sustained seizure-induced tachycardia produces micro-lesions, myofibre injury and significant fibrotic deposition throughout the subendocardium as early as 48 hour. This damage can lead to the development of substrates of arrhythmogenesis and left ventricular dysfunction.

The evidence of dilated cardiomyopathy and an increased susceptibility to arrhythmias, presented here, may be implicated in the development of sudden cardiac death in epileptic patients. Prophylactic therapy with clonidine, reduced both seizure severity and cardiac dysfunction following systemic KA. However studies with clonidine therapy, were not continued in this thesis as atenolol produced superior cardiac protection without the sedative side effects of the α2 agonist. Both prophylactic and intervention with atenolol has consistently proved to be an effective therapy in this study. Interestingly, atenolol intervention therapy provided neuroprotection during seizure. This action has been postulated to result as a consequence of preserved cardiac function and oxygen supply to the brain (Figure 7.6). Atenolol use has been reported in clinical trials in patients with epilepsy (McKee et al., 1994), although disappointingly there is limited information
on how β–blockade influences seizure. Mayer and Spechy (1995) reported reduced seizure frequency in patients with startle-induced seizures following propranolol administration. Atenolol was shown to effectively prevent QT syndrome in cases presenting as epilepsy (Pacia et al., 1994). In a study assessing seizure-induced heart rate changes, Rugg-Gunn et al. (2004) reported severe bradycardia (30 b.p.m. for 15 seconds) in a patient on atenolol. Conversely, atenolol has been proven to effectively reduce the development of stress-induced cardiomyopathy and should be considered for prophylactic administration in at-risk situations (Cruickshank et al., 1988; Littlejohn et al., 2008; Celano et al., 2011; Marabotti et al., 2014).

Intervention therapy with diazepam, significantly reduced the seizure behaviours and prevented hippocampal damage. However, in this study diazepam did not provide any cardioprotection, suggesting that preventing the seizures with antiepileptics may not provide sufficient cardiac protection during seizure activity. Combination therapy with atenolol and diazepam, significantly protected the heart and brain during seizure (Figure 7.6). Diazepam and atenolol intervention reduced seizure behaviours, preserved EEG function and decreased hippocampal apoptosis and inflammation. Equally cardiac indices of injury (HR, QTc interval, T wave amplitude, BP and HRV) were also attenuated back to baseline levels. Furthermore, combination therapy reduced the extent of left ventricular dysfunction, myocardial injury and arrhythmia susceptibility. These results clearly indicate the benefit of atenolol in combination with antiepileptic mediation in preserving cardiac function consequent to seizures.
In summary, this thesis strongly recommends trialling the clinical use of cardioprotective drugs with antiepileptic agents, to preserve cardiac function and reduce the risk of arrhythmias during seizures. Atenolol is generally clinically well tolerated and therefore may be considered as a prophylactic therapy in epilepsy. As atenolol was shown in this study to enhance the anticonvulsant effect of diazepam, a clinical study investigating the use of atenolol in combination with antiepileptic medication may provide therapeutic value in patients with refractory epilepsy (30% of epileptics). The functional cardiac results obtained in this study bear a strong resemblance to clinical data and clearly show that ECG abnormalities and structural heart damage occur rapidly after the onset a single seizure event. Our study highlights the importance of protecting the myocardium with sympatholytics during seizure.

**Figure 7.6.** Summary of study and proposed mechanism of seizure-induced cardiomyopathy and intervention therapy. VSE: Vagal sympathetic effect; EF: Ejection fraction; FS: Fractional shortening; LVID: Left ventricular internal dimension; EV: end volumes; NA: noradrenaline.
Chapter 8

Appendices
8.1. Supporting data for intrahippocampal KA studies

8.1.1. Determining the dose of intrahippocampal KA

Intrahippocampal KA (0.5-2 nmol) resulted in an immediate increase in seizure behaviours, where animals exhibited squinting, mastication, head tremors and WDS (Figure 8.1.1 and 2). Administration of 0.5, 1 or 2 nmol of KA produced an average behavioural score of 1.85 ± 0.4, 2.1 ± 0.6 and 3.2 ± 0.2, respectively. There was also a significant dose-dependent increase in the number of WDS (218-278) and Level 4 (52-204) behaviours, respectively. No Level 5 behaviours were recorded in the 0.5 or 1 nmol group while one of the five 2 nmol rats displayed Level 5 behaviours.

Administration 0.5 and 1 nmol of KA resulted in a small increase in HR to 397.7 ± 24.3 b.p.m. and 419.3 ± 32.7 b.p.m. (Figure 8.1.3). 2 nmol of KA resulted in an immediate increase in HR to 481.2 ± 25.9 b.p.m. and remained elevated until 98 minutes ($P<0.05$ compared to baseline) with an mean HR of 454.7 ± 24.2 b.p.m. No change in the QTc or T wave amplitude were observed at 0.5 and 1 nmol, however significant increases were observed following administration of 2 nmol (34-46 and 58-68 minutes, Figure 8.1.4 and 8.1.5). The T wave amplitude in the 2 nmol group increased by 21-26% 16-78 minutes post-KA ($P<0.05$ compared to baseline).

As seizure severity and ECG changes increased so did the extent of cardiac damage observed at 48 hours. There was a dose-dependent increase in the extent of morphology observed in KA treated hearts (Figures 8.1.6) with fibrosis, oedema and hypercontracture band necrosis observed in a majority of 2 nmol hearts. All KA treated hearts had evidence of inflammatory cell infiltration and there was a dose-dependent increase in oedema, reaching significance at 2 nmol.

![Figure 8.1.1. Effect of intrahippocampal saline or KA administration (0.5-2 nmol, 1 ug/min) on behavioural scores in rats (n=5/group). Cumulative behavioural score represents the sum of maximum score recorded every minute. The number of wet dog shake (WDS) and Level 4 behaviours were also recorded over the 180 min period following KA. # $P<0.05$ compared to baseline. * $P<0.05$ compared to 2 nmol.](image)
Figure 8.1.2. Effect of intrahippocampal saline or KA (dashed line) administration (0.5-2 nmol, 1 μg/min) on behavioural scores in rats (n=5/group). Each point represents the mean maximum score every 5 minutes, # P<0.05 compared to baseline.

Figure 8.1.3. Effect of intrahippocampal saline or KA administration (0.5-2 nmol, 1 μg/min) on heart rate (beats per min, b.p.m) in rats (n=5/group). Data was analysed as one minute on and one minute off. # P<0.05 compared to baseline.
Figure 8.1.4. Effect of intrahippocampal saline or KA administration (0.5-2 nmol, 1 μg/min) on QTc interval (ms) in rats. Data was analysed as one minute on and one minute off. *P<0.05 compared to baseline.

Figure 8.1.5. Effect of intrahippocampal saline or KA administration (0.5-2 nmol, 1 μg/min) on T wave amplitude (%) in rats. Data was analysed as one minute on and one minute off and normalised to baseline at time zero. *P<0.05 compared to baseline.
Figure 8.1.6. Micrographs of MSB stained LV subendocardium in rats 48 hours following KA. A) Normal representative myocardium. B) Myocyte vacuolisation, indicating reversible ischaemic damage. C-E) Inflammatory necrosis encapsulated by collagen deposition (blue fibres) indicative of early restorative fibrosis. E) Fibrosis and oedema. F) Hypercontraction band necrosis associated with fibre derangement which can be caused by elevated catecholamine levels. G) Quantification of fibrosis and oedema.
8.1.2. Drug Cannula placement
Placement of the drug cannula was confirmed (n=20) by injecting 2 μL of Evan’s Blue dye into the right hippocampus (Figure 8.2.1). Placement was found to be consistent in all rats at this age and weight range.

Figure 8.1.7. Image of the cannula placement (A; Paxinso and Watson, 2007) and photos confirming correct cannula placement following dye administration (B and C).
8.1.3. Correlations

This section explores the relationship between heart and brain indices. There is association between mean seizure behavioural score and heart rate over the course of the 180 minutes post intrahippocampal KA (Figure 8.1.8). This correlation is stronger ($R^2 = 0.56$) when diazepam was removed from the analysis, due to the potential seizure-independent changes in HR. As HR increases, there is an increase in the extent of cardiac and hippocampal ApopTag positive cells (Figure 8.1.9 and 10).

**Figure 8.1.8.** Correlation between heart rate and behavioural score over the 180 minutes following intrahippocampal KA. (A) Represents all treatment groups, while (B) excludes diazepam treated animals.

**Figure 8.1.9.** Correlation between heart rate and cardiac fibrosis (A) or apoptosis (B) over the 180 minutes following intrahippocampal KA

**Figure 8.1.10.** Correlation between hippocampal apoptosis (ApopTag positive cells) and mean heart rate (A) or mean behavioural score (B) over the 180 minutes following intrahippocampal KA
8.2. Effect of pre-treatment with diazepam prior to kainic acid

Diazepam (5 mg/kg, sc) was administered prior (10 minutes) to seizure induction to examine whether the bradycardia was a consequence of seizure generation or KA acting directly on the heart. Diazepam pre-treatment completely prevented seizure induction as determined by EEG and behavioural analysis (Figure 8.2.1A), although there was no significant difference in HR between saline and diazepam pre-treated animals (Figure 8.2.1B).

![Figure 8.2.1](image.png)

**Figure 8.2.1.** The effect of diazepam or saline administration (black line) prior to seizures induced by subcutaneous KA (dashed line). EEG power and behavioural score was presented as the mean over the 180 minutes post-KA (A). Heart rate was analysed every minute over the course of the study (B; not significantly different). "P<0.05 compared to baseline, *P<0.05 compared to saline pre-treated animals.

8.3. Prophylactic treatment with atenolol prior to intrahippocampal KA

Prophylactic treatment with atenolol prior to subcutaneous KA offered significant cardiac protection during SE (Chapter 4). This section aimed to determine if atenolol prophylactic treatment would offer the same benefit in the intrahippocampal model of seizure activity. Pre-treatment with atenolol for three days prior to seizure-induction (5 mg/kg, sc), resulted in a significant decrease in behavioural score and heart rate over the 180 minute period compared to the saline pre-treated animals (Figure 8.3.1). Left ventricular micrographs from atenolol pre-treated animals, showed normal morphology at 48 hours post seizure-induction (Figure 8.3.2).
Figure 8.3.1. Effect of saline or atenolol pre-treatment for three days prior to intrahippocampal KA administration (dashed line) on behavioural score (A) or heart rate (B) over 180 minutes.

Figure 8.3.2. Representative left ventricular micrographs from control naïve rats compared to saline and atenolol pre-treated seizure animals at 48 hours post-KA.
8.4. Comparison of subcutaneous and intrahippocampal KA administration

Systemic administration of KA produced a period of hypoactivity coinciding with bradycardia, however, 30-60 minutes post KA seizure activity progressively increased (Figure 8.4.1 and 2). These high level seizure behaviours produced significant increases in HR, QTc prolongation and T wave elevation. Intrahippocampal KA appeared to be a better model of epileptic activity, animals immediately had high level seizure behaviours although there were periods of intermittent normal behaviours. Behavioural activity in the intrahippocampal was significantly higher at 24 and 48 hours than in the subcutaneous model. Intrahippocampal KA produced an immediate a sustained increase in HR (Figure 8.4.1). There was no significant difference in structural damage at 48 hours following subcutaneous or intrahippocampal KA (Table 8.4.1).

Figure 8.4.1 Behavioural and heart rate following subcutaneous (10 mg/kg) or intrahippocampal (2 nmol) KA.

Table 8.4.1. Relative extent of morphological features at 48 hours post-seizure onset in left ventricular subendocardial regions.

<table>
<thead>
<tr>
<th></th>
<th>10 mg/kg, sc</th>
<th>2 nmol, ih</th>
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<tbody>
<tr>
<td>Hypercontracture band necrosis</td>
<td>6.2 ± 1.1</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>Myocyte Vacuolisation</td>
<td>7.7 ± 1.1</td>
<td>7.4 ± 0.7</td>
</tr>
<tr>
<td>Oedema</td>
<td>31 ± 5%</td>
<td>16.3 ± 1.5%</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.9 ± 0.3%</td>
<td>0.8 ± 0.1%</td>
</tr>
</tbody>
</table>

Quantification of each feature was conducted in a double-blind manner on 10 sequential fields from each ventricular section. Contraction band necrosis in each field was positively graded on the presence of 3 myocardial cells containing contraction bands. Reversible nuclear vacuolisation was graded on the presence of ≥2 vacuolised nucleus in each field. No significant differences. Fibrosis and oedema were quantified using Photoshop, to count the number of negative (white, oedema) and positive (blue, fibrotic) pixels.
Figure 8.4.2. Representative EEG (grey trace) and behavioural scores (black plot) following KA administration (10 mg/kg sc compared to 0.5, 1 or 2 nmol of intrahippocampal KA, dashed line). Expanded images represent EEG (10 seconds) traces with corresponding behavioural response.
8.5. Blood Brain Barrier

Blood brain barrier (BBB) permeability was measured by injecting $^{14}$C-Sucrose ($4 \, \mu$Ci, iv) into halothane anaesthetised rats, 1 or 3 hours post subcutaneous (10 mg/kg) or intrahippocampal (2 nmol) KA administration (protocol adapted from Friden et al., 2010). Brain tissue was weighed as either ipsilateral or contralateral hemisphere and submerged in 10 ml/g deionised water prior to homogenisation at 1300-1500 r.p.m. by a Heidolph homogeniser. 1 ml of homogenised tissue was pipetted into the scintillation vial with 3 ml scintillant fluid (UltimaGold, PerkinElmer, USA) was added. Radioactivity was measured in duplicate and counted as disintegrations per minute (DPM) in the solution over 1 hour per sample. There was no significant increase in the extent of $^{14}$C-Sucrose into the brain following subcutaneous KA administration (Figure 8.5.1). Intrahippocampal KA administration significantly increased the permeability of the BBB, with a 3-fold increase in the uptake index measured at 3 hours (Figure 8.5.1). Mannitol (25% w/v saline, 0.25 ml/sec/kg for 20 sec, 37°C) was used to as a positive control to confirm that $^{14}$C-Sucrose radioactivity was an accurate way to measure breakdown of the BBB (work performed in collaboration with William Fulton).

![Figure 8.5.1](image.png)

**Figure 8.5.1.** The amount of $^{14}$C-Sucrose in the ipsilateral and contralateral brain 1 or 3 hours following KA administration (10 mg/kg sc or 2 nmol ih). Data presented as uptake index which accounts for rat weight. Uptake index is determined as (disintegrations per minute (DPM) count/brain weight)/(injected DPM/Rat weight).
8.6. Dose determination

The dose of intervention therapy used in the present study, was determined using the dose translation equation (human to rat) described by Reagan-Shaw et al. (2007; Table 8.6.1). Atenolol (50-100 mg) is used to treat hypertension, angina, arrhythmias and post-myocardial infarction (Medsafe, 2010). This is equivalent to 0.7-1.4 mg/kg assuming 70 kg human which suggests that 5 mg/kg in a rat is an appropriate dose to preserve cardiac function.

Intravenous diazepam administration generally does not exceed 30 mg (0.42 mg/kg assuming 70 kg patient; Medsafe, 2012) which is lower than the dose used in the present study. Diazepam administration had a rapid anticonvulsant effect, as it is taken up into the brain within 5 minutes (Friedman, 1986). However, due to its high lipophilicity diazepam is rapidly redistributed to fat stores and is rapid eliminated resulting in a short duration of action (Friedman, 1986; Chapman, 2001). A dose of 5 mg/kg, was used in this study as it has been shown to effectively reduce seizure severity in rats (Loscher & Schwark 1985; Baran et al., 1994). Diazepam can be used for short-term management of some types of epilepsy, with doses of 2-60 mg daily in divided dosages (0.03-0.9 mg/kg assuming 70 kg patient). Therefore, the maintenance dose of 1 mg/kg twice daily was chosen for the present study.

Table 8.6.1. Human estimated dose calculation as described by Reagan-Shaw et al. (2007).

<table>
<thead>
<tr>
<th></th>
<th>Dose</th>
<th>Dose Translation from Animal to Human Studies based on surface area.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>5 mg/kg</td>
<td>Human estimated dose = 5 mg/kg x (6/37) = 0.82 mg/kg.</td>
</tr>
<tr>
<td>Diazepam</td>
<td>5 mg/kg</td>
<td>Human estimated dose = 5 mg/kg x (6/37) = 0.82 mg/kg.</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg, twice daily</td>
<td>Human estimated dose = 1 mg/kg x (6/37) = 0.16 mg/kg. Similar to clinical studies.</td>
</tr>
</tbody>
</table>
Chapter 9

Reference List


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